Identification of genes encoding β-ketoadipate pathway enzymes in biodegradation of aromatic compounds by *Aspergillus niger*

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ABSTRACT

Identification of genes encoding β-ketoadipate pathway enzymes in biodegradation of aromatic compounds by *Aspergillus niger*

Farnaz Olyaei

Fungi, together with bacteria, are responsible for the degradation of aromatic compounds in the environment. The β-ketoadipate pathway is a common pathway for the cleavage of dihydroxylated aromatics such as catechol but while the bacterial enzymes for this pathway have been well-characterized, those from fungi have not.

This pathway in bacteria includes two branches: one with six enzymes to convert catechol to acetyl coenzyme A and succinate, and the other with seven enzymes to catalyze the conversion of protocatechuate (3,4-dihydroxybenzoate) to the same products plus CO_2. BLAST sequence comparisons identified genes in the *Aspergillus niger* genome that are likely to encode these enzymes. *Aspergillus niger* cDNA was used to amplify 12 of these genes, which were subsequently cloned using ligation-independent cloning into the pLATE11 vector and the resulting plasmids were tested for expression in *E.coli BL21*. Of these, 4 proteins were successfully expressed and then purified using a combination of column chromatography techniques. Assays of two of these proteins combined with UV analysis of reaction intermediates indicated that they encode β-carboxymuconate cyclase (NRRL3_02586) and β-carboxymuconolactone hydrolase/decarboxylase (NRRL3_01409) activities from the protocatechuate branch of the pathway. The β-carboxymuconolactone hydrolase/decarboxylase was shown to be a monomer and is the first such enzyme to which a sequence has been assigned. The other two proteins, encoded by NRRL3_10507 and NRRL3_04788, were active in assays for muconate isomerase and 3-oxoadipate enol lactone hydrolase, respectively, from the catechol branch of the pathway. Both enzymes were found to be dimers. In comparison, the bacterial muconate isomerase is a decameric enzyme with smaller subunits, while bacterial 3-oxoadipate enol lactone hydrolase is also dimeric.

These data have resulted in the identification of molecular functions for four genes encoding key enzymes in aromatic degradation by *A. niger*.

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1 Introduction

1.1 Aromatic compounds in the environment:

Aromatic compounds are one of the largest and most diverse groups of chemicals in the environment. Although the three aromatic amino acids phenylalanine, tyrosine and tryptophan, as well as their derivatives, may be found in all organisms, aromatics are mainly produced by plants, where they may be found in secondary metabolites with diverse structure, as well as in the aromatic polymer lignin. Moreover, by industrial activity, synthetic aromatics, many of which are petroleum derivatives, can enter the environment.

Some of the simplest of these chemicals are shown in Figure 1.1.

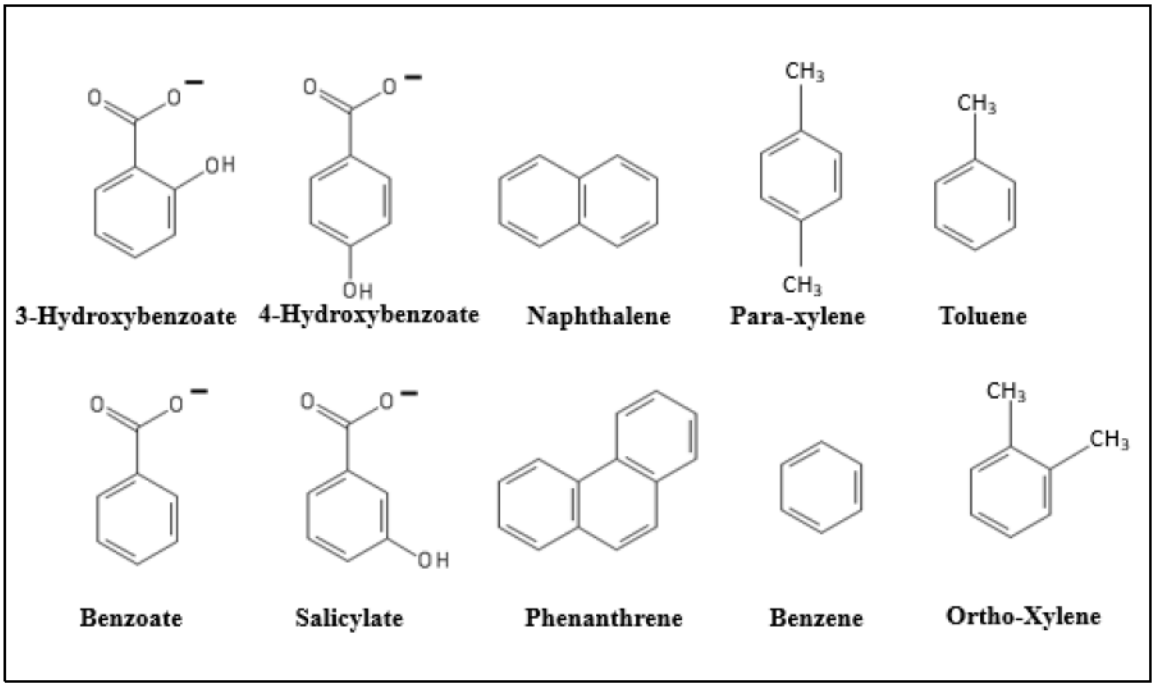


Figure 1.1: Examples of natural and synthetic monocyclic and polycyclic aromatic compounds and their structures[1, 2, 3].

The high resonance energy of the aromatic ring gives aromatic compounds an unusual stability and makes them less susceptible to degradation in the environment. Since some of these compounds have been reported to be toxic and carcinogenic in higher organisms, their degradability is of interest. Humans and animals have a limited capacity to process aromatic compounds. Their involvement in primary metabolism in these organisms is limited to metabolism of the three aromatic amino acids and

their derivatives. If they cannot be degraded, they may be subject to detoxification reactions in mammals, involving cytochrome P450 enzymes which can hydroxylate the aromatic ring and convert them to some secondary metabolites such as phenols and ketones[4]. Given their toxicity and limited degradation in higher organisms, and the necessity to recycle them in the carbon cycle, their degradation by microorganisms such as bacteria and fungi[5] in the environment is of scientific interest and concern[6].

1.2 Degradation of monocyclic aromatic compounds by microbes

Biodegradation of aromatic compounds by bacteria and fungi mostly occurs in one of two ways depending on whether O_2 is present or not:

a. Anaerobic: In anaerobic environments, microorganisms use nitrate, sulfate, ferric ions, CO_2 or other electron accepters to oxidize and destabilize the aromatic ring and then degrade the resulting products. In one anaerobic pathway, benzoate is converted to the activated form, benzoyl-CoA as a major metabolite, and then this product is reduced by benzoyl-CoA reductase to cyclohexa-1,5-diene-1-carboxyl-CoA (1,5-dienoyl-CoA) which will participate in further enzyme-mediated degradation reactions and produce acetyl-CoA molecules[1, 5, 6, 7, 8].
b. Aerobic: oxygen is used as terminal electron acceptor and for hydroxylation and/or dioxygenation of the benzene ring that precedes additional enzyme-catalyzed reactions that convert the ring cleavage product to central metabolites[1, 9, 10].

For the purpose of this thesis only aerobic microbial catabolism of aromatics is reviewed, since the pathways under study occur in the presence of oxygen. Furthermore, the compounds under study are all monocyclic so polycyclic aromatics are not discussed further.

1.3 Aerobic degradation of monocyclic aromatic compounds

The microbial processes for degradation of monocyclic aromatics have been most intensively studied in bacteria. Pathways converge on common dihydroxylated substrates for ring cleavage dioxygenases. In these processes, oxygenases (either monooxygenases or dioxygenases) activate O_2 and react it in ways that lead to production of di-hydroxylated benzene rings. Dihydroxylated rings with the hydroxyls either *ortho* or *para* to each other are substrates for ring cleavage dioxygenases[11]. Two common

dihydroxylated products for ring cleavage dioxygenases are catechol (1,2-dihydroxybenzene) and protocatechuate (3,4-dihydroxybenzoate), whose metabolism by the bacterium Pseudomonas putida was originally extensively studied by Ornston and colleagues[12, 13, 14, 15]. Ring cleavage enzymes may generally be classified as either intradiol dioxygenases or extradiol dioxygenases depending on how they cleave the aromatic ring[1, 16]. Intradiol dioxygenases cleave the ring molecule between the hydroxyl groups, which results in "*ortho* cleavage" (also called "intradiol cleavage") and extradiol dioxygenases cleave the ring adjacent to one of the hydroxyl groups and results in "*meta* cleavage" (also known as extradiol cleavage")[9, 10] (Figure 1.2). Depending on the species and the growth substrate, bacteria can cleave catechol and protocatechuate either by intradiol or extradiol cleavage, and the metabolism of the resulting ring cleavage products will each be degraded by characteristic suites of enzymes to Krebs cycle or glycolytic intermediates[9, 10].

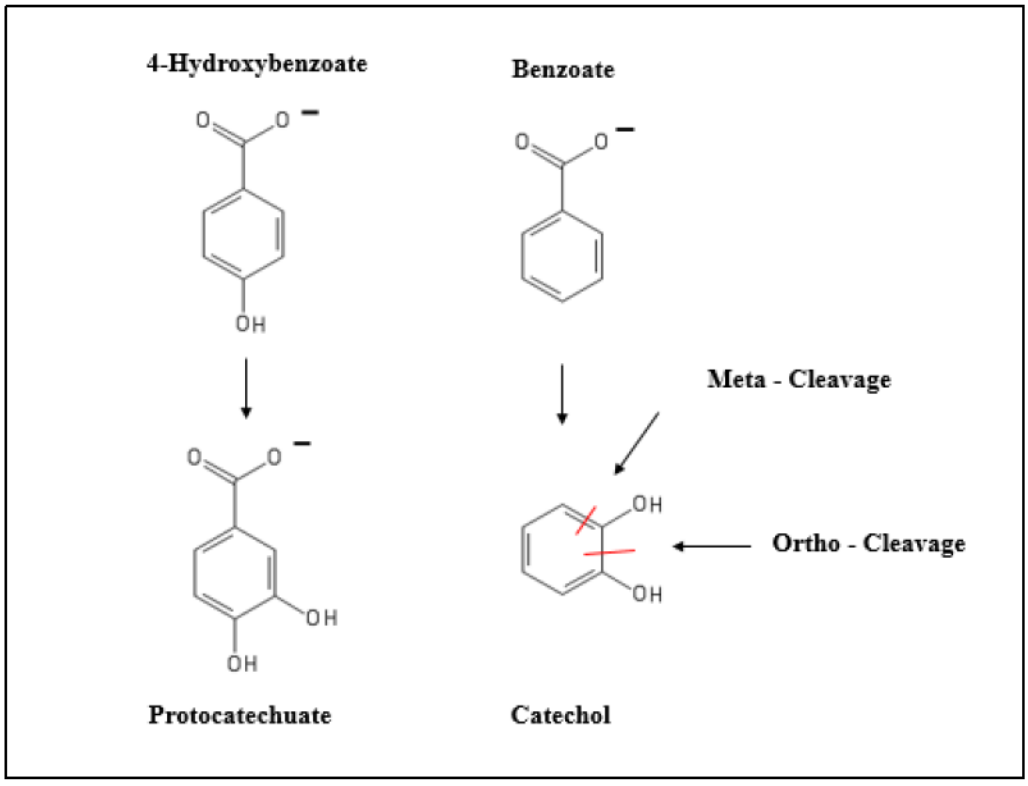


Figure 1.2: Aromatic ring cleavage for two common ring cleavage substrates, produced from p-hydroxybenzoate and benzoate[1]

The rest of this *Introduction*, and the topic of the research in this thesis, is concerned with the *ortho*-cleavage of catechol and protocatechuate, which although they are cleaved by dioxygenases of different

specificity, catechol 1,2-dioxygenase and protocatechuate-3,4-dioxygenase, respectively, are eventually converted by separate pathways to β-ketoadipate. Thus, the corresponding catabolic pathways for these two compounds are referred to as the two branches of the "β-ketoadipate pathway"[2].

1.4 The β-ketoadipate pathway in bacteria

This pathway is distributed among a range of microorganisms, but it has been most extensively studied in bacteria. Although the involvement of β-ketoadipate in degradation of certain aromatics had been demonstrated as early as the 1950's in both bacteria[12, 13, 14, 15] and fungi[17, 18, 19], the biochemistry, chemistry, enzymology and genetics of the pathway were worked out in detail during the 1960s and 1970s in the bacterium *Pseudomonas putida* by L Nicholas Ornston and his colleagues[12, 13, 14, 20].

1.4.1 Catechol branch of the β-ketoadipate pathway in bacteria

In this branch (Figure 1.3 c) catechol, the ring cleavage substrate, is converted to *cis,cis*-muconic acid by catechol 1,2-dioxygenase[14, 18, 21]. Next, muconate cyclase lactonizes this metabolite to muconolactone. This product is then transformed to 3-ketoadipate enol lactone by muconolactone isomerase through an endocyclic rearrangement of the double bond of muconolactone to create β-ketoadipate enol-lactone[22]. Following that, β-ketodipate enol lactone hydrolase cleaves β-ketoadipate enol-lactone by hydrolysis to β-ketoadipate[14, 18].

1.4.2 Protocatechuate branch of the β-ketoadipate pathway in bacteria

In this branch in bacteria (Fig. 1.3 b), protocatechuate is cleaved to β-carboxy-*cis,cis*-muconic acid by protocatechuate 3,4-dioxygenase[14, 18]. This metabolite is converted to β-carboxymuconolactone through gamma-lactonization by a *syn*-1,2 addition-elimination reaction: the responsible enzyme is β-carboxymuconate cyclase. By decarboxylation, β-carboxymuconolactone is converted by β-carboxymuconolactone decarboxylase to β-ketoadipate enol-lactone and then, similar to the catechol pathway, by hydrolysis of 3-oxoadipate enol-lactone by β-ketoadipate hydrolase, β-ketoadipate is produced.

1.4.3 Enzymology of the β-ketoadipate pathway in bacteria

All of the enzymes involved in the β-ketoadipate pathway in *P. putida* have been purified to homogeneity and characterized at least to some extent. Much of this work was done by Ornston and colleagues during the course of their pioneering work on the biochemistry of the pathway: this includes β-carboxymuconate lactonizing enzyme, muconate lactonizing enzyme, β-carboxymuconolactone decarboxylase, muconolactone isomerase and β-ketoadipate enol-lactone hydrolase[12, 13, 14]. Others were described elsewhere, including: protocatechuate 3,4-dioxygenase, catechol 1,2-dioxygenase and β-carboxy muconolactone hydrolase[18, 23, 24].

1.4.3.1 Lactonizing enzymes

The bacterial lactonizing enzymes share some properties. The pH optimum of both the β-carboxymuconate lactonizing enzyme and the muconate lactonizing enzyme is reported to be between pH 6 and pH 8. Also, phosphate ion could inhibit the activity of these enzymes and both enzymes are stable at 60°C for about 30 min[13].

In the other hand they are different in terms of their molecular weight and Michaelis constant. The molecular weight for β-carboxymuconate lactonizing enzyme has been reported to be 190,000 whereas for the muconate lactonizing enzyme has been reported to be 220,000. The β-carboxymuconate lactonizing enzyme Michaelis constant is reported to be 7.5×10^{-5} M and for muconate lactonizing enzyme is reported to be 1.0×10^{-4} M. Both $MgCl_2$ and $MnCl_2$ stimulate of the muconate lactonizing enzyme activity[20, 22]. Although these enzymes share some properties, they are not able to lactonize the substrates of each other.

1.4.3.2 β-carboxymuconolactone decarboxylase, muconolactone isomerase

The molecular weights of both enzymes are estimated to be 93000 Daltons and they convert their substrates to the β-ketoadipate enol-lactone through a similar mechanism. Also, their Michaelis constant is reported to be 8.2×10^{-4} M. But they do not have any activity for the substrate of the other enzyme[20, 22].

1.4.3.3 β-ketoadipate enol-lactone hydrolase

The optimal activity of this enzyme was reported to be between pH 7.5 and pH 10. The Michaelis constant for β-ketoadipate enol-lactone hydrolase Michaelis constant is reported to be 1.2×10^{-s} M. The molecular weight of this enzyme is reported to be 33,000[20, 22].

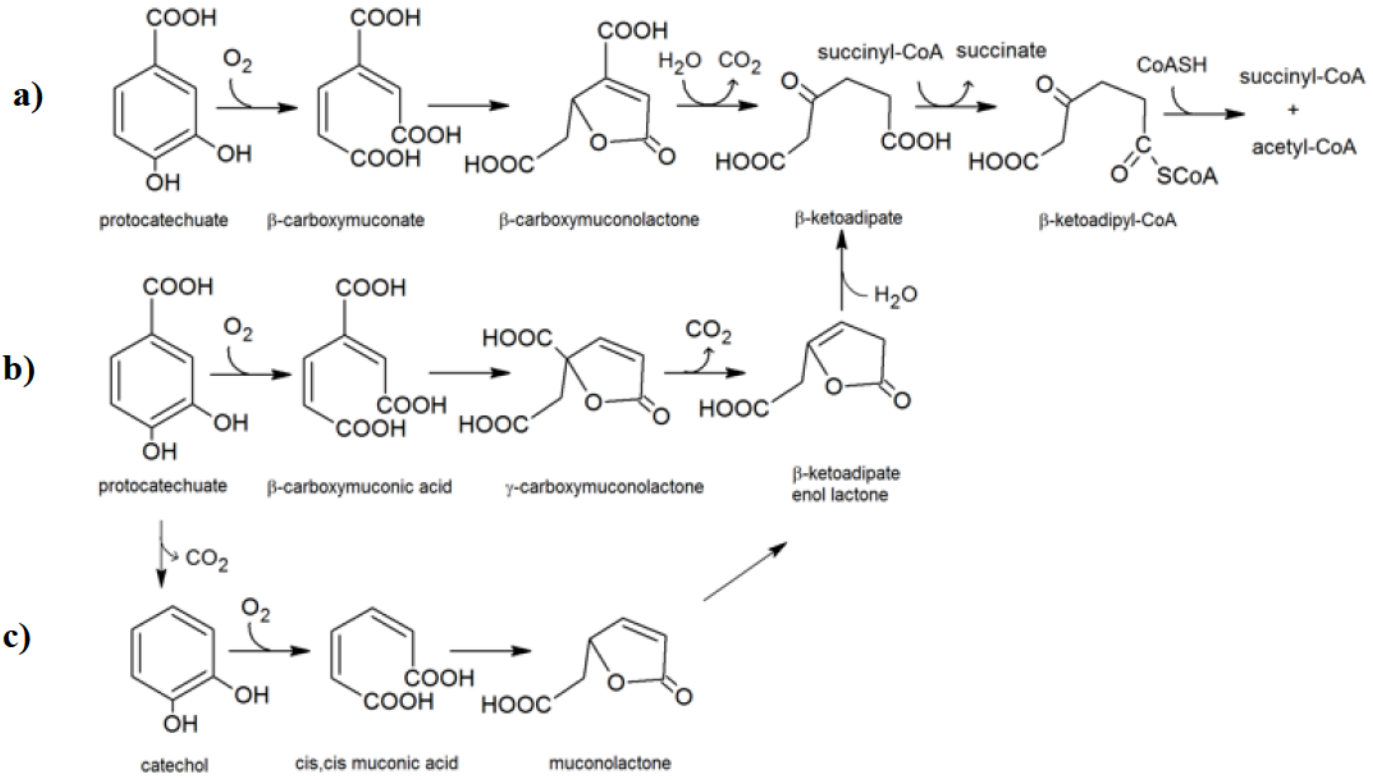


Figure 1.3: The β-ketoadipate pathway in fungi and bacteria; a) protocatechuate branch in bacteria and fungi, b) bacterial protocatechuate branch, c) fungal catechol branch. Used by permission of Dr. J. Powlowski.

1.4.4 Genetics and regulation of the β-ketoadipate pathway in bacteria

The genetics and regulation of the β-ketoadipate pathway have been extensively studied in bacteria, where the pathway is chromosomally encoded in *Pseudomonas putida*. Genes involved in the catechol branch (*cat* genes) and protocatechuate branch (*pca* genes) in benzoate and 4-hydroxybenzoate degradation have been identified[25, 26, 27, 28, 29]. *Cat* genes include *catA*, encoding catechol 1,2-dioxygenase, *catB* which encodes *cis,cis*-muconate lactonizing enzyme and *catC* that encodes muconolactone isomerase. It has been reported that the *pca* genes are arranged in four clusters *pcaHG, pcaBDC, pcaIJ, and pcaF*[26, 27, 29]. Most of these genes are regulated by *pcaR* as an inducer. The regulatory enzyme in protein expression of *pcaHG,* encoding protocatechuate 3,4-dioxygenase, has not been reported. Expression of this gene is induced by protocatechuate. The *pcaBDC* cluster includes genes encoding β-carboxymuconate lactonizing enzyme (*pcaB*), β-ketoadipate enol-lactone hydrolase (*pcaD*), and β-carboxymuconolactone decarboxylase (*pcaC*). The *pcaIJ* cluster includes genes encoding β-ketoadipate: succinyl-coenzyme A, and β-ketoadipate: succinyl-CoA transferase[30]. The positions of

cat genes are at 4236-4239 kb of the *P. putida KT2440* genome, and for *pca* genes are at 1566-1575 kb for *pcaRKFTBDCP*, at 4457–4459 kb for *pcaIJ* and at 5281–5282 kb for *pcaGHr*[31] .

1.5 The β-ketoadipate pathway in *A. niger*

In general, it has been reported that *A. niger*, a filamentous ascomycete fungus, can grow on a wide range of aromatics such as benzoate and its derivatives, protocatechuate, catechol, vanillate, ferulate, and caffeate[18]. A few of its enzymes and activities, likely involved in aromatic degradation allowing growth on some of these compounds, have been purified, and their characteristics have been identified. These enzymes include benzoate hydroxylase[32], p-hydroxybenzoate hydroxylase[33] and 2,3-dihydroxybenzoate decarboxylase[34]. Several enzymes of the β-ketoadipate pathway, have been purified and to some extent characterized, as described below. However, with the exception of benzoate hydroxylase, the genes encoding these enzymes have not been identified with certainty.

RB Cain *et al.*in the 1960s and 1970s studied many of the metabolites and enzyme activities of the protocatechuate branch of the β-ketoadipate pathway in *A. niger* [18, 23, 24]. The known biochemical details of the pathways in bacteria and *A. niger* are similar in many respects, but not all. The biggest difference is that in *A. niger* and some other fungi[17, 18], following intradiol cleavage of protocatechuate, β-carboxymuconate cyclase converts β-carboxymuconic acid to β-carboxymuconolactone (Figure 1.3c), rather than $_{\gamma}$-carboxymuconolactone (Figure 1.3b). This metabolite then undergoes decarboxylation followed by hydrolysis apparently using only one enzyme with both activities: β-carboxymuconolactone hydrolase[18, 23, 24] (Figure 1.3.c). On the other hand, the enzyme activities of the catechol branch of the β-ketoadipate pathway were not reported in these studies. Some studies on this branch have been done on *Trichosporon cutaneum* using phenol and resorcinol as a source of carbon by Gaal *et al.* They have reported two enzymes are involved in the delactonizing as in bacterial[19].

Some muconate lactonizing enzymes (MLE) of the β-ketoadipate pathway from different fungal species have been studied and compared with their bacterial counterparts. These studies focused on differences between prokaryotic and eukarytic MLE enzymes in terms of their 3D structure and mechanism. The result of studies on MLE from *Trichosporon cutaneum*[35, 36, 37] and CMLE from *Neurospora crassa*[38, 39] reveal that eukaryotic cycloisomerases are not related to the prokaryotic cycloisomerases and they have a unique structural motif in eukarotic microorganisms.

Also, a distinguishable substrate specificity has been reported for these enzymes[36, 37].

Three classes of MLE exist:

1. The prokaryotic MLEs with TIM barrel: these enzymes are related to mandelate racemase[39, 40, 41].
2. The prokaryotic CMLEs related to the fumarase class II family[38, 39].
3. Eukaryotic MLEs and CMLEs which are genetically related to each other[38]. These enzymes do not need metal cofactors for their activity. This is similar to prokaryotic CMLEs, but they catalyze a syn addition reaction similar to prokaryotic MLEs.

In terms of the structure, prokaryotic MLE and CMLEs contain an α/β barrel where as eukaryotic CMLEs contains a seven-bladed β propeller[39, 40].

1.6 Assignment of β-ketoadipate pathway enzyme functions to *A. nidulans* genes based on genomic and proteomic analyses

In a recent study, the sequences of *A.nidulans* genes encoding enzymes involved in the β-ketoadipate pathway were proposed based on genomics and proteomics analyses[21]. In that study *A. nidulans* mycelial and extracellular proteins were analysed and identified using two-dimensional gel electrophoresis (2DE) and ProteinPilot software. Some gene replacement mutants were prepared and the mutants were grown on solid and liquid media supplemented with acetate (as a negative control), benzoate or salicylate as the carbon sources. The growth of the mutants was monitored in order to assign the essential genes in both catechol (used by salicylate) and protocatechuate (used by benzoate) branches[21]. These assignments were used in the course of this study to help identify possible orthologues in *A. niger* to target for expression and purification, for further study and characterization at the enzyme level. In some cases, bacterial gene sequences encoding β-ketoadipate pathway enzymes were also used.

1.7 The goal of this study

The present study aims to identify genes encoding enzymes involved in the downstream reactions of the β-ketoadipate pathway in the model fungus *A. niger*, and to unambiguously assign functions based on expression and direct assays of enzyme activity. The genome sequences of many different strains of *A. niger* have been reported and currently are available, for example of *A. niger* ATCC 1015v4.0[42] (Joint Genome Institute), and *A. niger* NRRL3[43]. This aids identification of potential genes encoding enzymes involved in the β-ketoadipate or related catabolic pathways. The

work reported here involves bioinformatics analyses, recombinant expression of selected genes, testing of expressed proteins for relevant enzyme activities and in some cases additional enzyme characterization. Assignment of molecular functions to specific gene products is expected to enable further study of the importance and relevance of the β-ketoadipate pathway in fungi.

2 Materials and Methods

2.1 Materials

Oligonucleotide primers were synthesized by Integrated DNA Technologies Inc. (Coralville, Iowa, USA). The forward primers included 5'-AGAAGGAGATATAACT-3'at the beginning and the reverse primers started with 5'-GGAGATGGGAAGTCA-3'for the cloning of the genes of interest into the pLATE11 vector of the Thermo Scientific aLICicator LIC cloning kit. The predicted Tm's for the primers were 65 ° C or greater and their GC contents were between 40-60%.

DNA sequencing was carried out at the McGill University and Genome Quebec Innovation Centre, Montreal, Quebec.

The GeneRuler 1 kb DNA Ladder (ThermoScientific) was used as the standard for agarose gel electrophoresis. The PCR fragment 6X loading dye buffer (ThermoScientific) was used to prepare samples for electrophoresis. The DNA polymerase used in PCR reactions was Phusion High-Fidelity DNA Polymerase (New England Biolabs (NEB)). For PCR reactions, the Applied Biosystems Verti 96 Well Thermal Cycler was used.

2.2 Gene finding and bioinformatics

Genes were identified by querying the *A. niger* genome repositories for two strains, ATCC 1015v4.0[42] (Joint Genome Institute), and the *A. niger* NRRL3 genome[43] with gene sequences from *A. nidulans*, *Pseudomonas putida and Acinetobacter calcoaceticus*. Oligonucleotide primers for PCR were designed based on the coding region of genes likely to encode the enzymes involved in the downstream reactions of the β-ketoadipate pathway.

2.3 PCR amplification and plasmid construction

A.niger cDNA from alfalfa-barley grown cells was provided by Annie Bellemare and Sandrine Marqueteau at the Centre for Structural and Functional Genomics (CFSG) and was used as a template to amplify genes. The PCR primers (Table 2.1) were designed according to the instructions from the manufacturer of the LICator LIC cloning kit (ThermoScientific)[44] . The forward primers started with the nucleotides 5'-AGAAGGAGATATAACT-3', and the reverse primers started with 5'-GGAGATGGGAAGTCA-3'.

Primer	Sequence (5'→3')
NRRL3_02586	AGAAGGAGATATAACTATGAAGCATCATCTTATGGTCGGTACCTGG
NRRL3_02586	GGAGATGGGAAGTCATTAATCATACCAAATTGCATTCATACCGAATCCC
NRRL3_00837	AGAAGGAGATATAACTATGCGTCTCCCCTACG
NRRL3_00837	GGAGATGGGAAGTCATCACTTCCTCTCAAGATCAGCCACCAG
NRRL3_08340	AGAAGGAGATATAACTATGACCAAAAAAGTCGAGGTTGACC
NRRL3_08340	GGAGATGGGAAGTCATTATGAGCGCTTGTACTCCCCATTC
NRRL3_10507	AGAAGGAGATATAACTATGACCTACACCATCACCTCCC
NRRL3_10507	GGAGATGGGAAGTCATCATTTCTTCCCACCCTGC
NRRL3_07569	AGAAGGAGATATAACTATGCCATATCTCACCCACAATGAAATTC
NRRL3_07569	GGAGATGGGAAGTCACTAAAGATTCGCTTTGGGTTTCCC
NRRL3_01886	AGAAGGAGATATAACTATGTCGCGGCCTGTTCC
NRRL3_01886	GGAGATGGGAAGTCATTACAGCATAGGCTTCAGATCAGC
NRRL3_01593	AGAAGGAGATATAACTATGGCAACCATTCGATTATCC
NRRL3_01593	GGAGATGGGAAGTCATCATTCCATGATCTCTCATTCC
NRRL3_11640	AGAAGGAGATATAACTATGCCGTCACAAGTGGC
NRRL3_11640	GGAGATGGGAAGTCACTACAGCATCGGCTTGACATC
NRRL3_01409	AGAAGGAGATATAACTATGGCGACCCCCGGC
NRRL3_01409	GGAGATGGGAAGTCACTAGTGCAGGTACTCATTCACCACCG
NRRL3_04788	AGAAGGAGATATAACTATGCCGACCGTCACC
NRRL3_04788	GGAGATGGGAAGTCATTACAGGGTTTGCACAAACC
NRRL3_11120	AGAAGGAGATATAACTATGACCGAACTAAGAATCACCAAAATCGAC
NRRL3_11120	GGAGATGGGAAGTCATCAATCATAACTAGCAACCGGCTC

Table 2.1: Primer Sequences used for gene amplification

PCR was performed using the Touchdown (TD) PCR technique[45], and the settings used to amplify all genes are shown in Table 2.2.

Phase 1	Step	Temperature	Time
1	Denature	95 ^{0}C	3 min
2	Denature	95 ^{0}C	30 s
3	Anneal	80 ^{0}C	45 s
4	Elongate	72 ^{0}C	60 s
Repeat steps 2–4 (10–15 times)			
5	Denature	95 ^{0}C	30 s
6	Anneal	72 ^{0}C	45 s
7	Elongate	72 ^{0}C	60 s
Repeat steps 5–7 (20–25 times)			
8	Elongate	72 ^{0}C	5 min
9	Halt reaction	4 ^{0}C	15 min
10	Hold	23 ^{0}C (room temperature)	Until removed from machine

Table 2.2:TD PCR parameters for gene amplification.

DNA fragments were separated by electrophoresis at 100 V on a 1 % agarose gel[46]. 30 µl Ethidium bromide was added in the gel to visualise the fragments. The bands of interest were cut out after identifying under Fotodyne UV illuminator using analytical mode and purified using the Roche High Pure PCR Product Purification Kit[47], then inserted into pre-linearized vector pLATE 11 from the aLICator LIC cloning kit, essentially as described by the manufacturer[44] .

2.4 Transformation of *E. coli*

The recombinant plasmids were transformed[48] into *E. coli* DH5α. A brief description of the procedure is as follows.

The cloning product (10 µl) was mixed with DH5α competent cells (50 µl) and incubated on ice for 30 min. Then, the mixture went through a heat-shock treatment by placing it at 42 °C for 45 sec and immediately incubated on ice for 2 min and then, followed by 1 hour of growth in LB at 37°C with 225 rpm shaking. The mixture centrifuged and the pellet plated on LB agar plates containing carbenicillin

(50 µg/ml), and grown overnight at 37 °C. The following day a single colony was selected and grown overnight at 37 °C in 5 ml LB medium containing 50 µg/ml carbenicillin. A plasmid miniprep kit[49] was used to purify the recombinant plasmids from the overnight culture essentially as described by the manufacturer.

The presence of the DNA inserts was confirmed by PCR using 5'- TAATACGACTCACTATAGGG -3' and 5'- GAGCGGATAACAATTTCACACAGG-3' LIC sequencing primers, as the forward and reverse PCR primers respectively which will add 200bp to the size of the amplified fragments. Moreover, the nucleotide sequences of the inserts were confirmed by DNA sequencing of the plasmids using the same primers as mentioned above.

2.5 Protein expression

The expression host was the *E. coli* BL21(DE3) strain[50]. This strain possesses the λDE3 lysogen, which expresses T7 RNA polymerase under the control of the lacUV5 promoter. The plasmid constructs harbouring genes predicted to encode muconolactone isomerase, β-ketoadipate enol lactone hydrolase, β-carboxymuconate cyclase and β-carboxymuconate hydrolase (plasmids are designated 10507-pLATE11, 04788-pLATE11, 02586-pLATE11 and 01409-pLATE11, respectively, where the number indicates the numbers in the NRRL3 gene identifier) were transformed into this strain as described in section 2.4 and plated on LB agar plates containing 50 µg/ml carbenicillin and grown overnight at 37 °C. The following day a single colony was selected and transferred to 50 ml liquid LB medium with 100 µg/mL ampicillin and incubated overnight at 37°C by shaking at 225 rpm. The optical density was monitored at 600 nm until it reached 0.5-1.0. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were incubated with shaking at 16 °C for 24 hours.

The cells were harvested by centrifugation at 7500 x g at 4 °C for 20 min, and the pellet was resuspended in buffer (2 ml/g cells) before making crude extract. The 10507-pLATE11 or 04788-pLATE11 transformants were resuspended using 50 mM HEPES buffer, pH 7.5; The 2586-pLATE11 cells were resuspended using 50 mM Tris-Cl buffer, pH 7.5; and 01409-pLATE11 cells were resuspended using 50 mM HEPES, pH 8.6, containing DTT 0.2 mM, and 10% glycerol.

2.6 Methodes of purification of enzymes

2.6.1 Preparation of crude extract

After the bacterial cell pellet was re-suspended in 50 mM HEPES buffer, pH 7.5, the cell suspension was sonicated using a BioLogics Model 300 VT ultrasonic homogenizer at 50% full power for 10 bursts of 10 s each. All procedures were carried out on ice. This was followed by centrifugation at 6000 x g for 1 h in order to prepare the cell-free extract. The supernatant (crude extract) was separated from the pellet and carried on to the next step.

In case of β-carboxymuconate cyclase, 50 mM Tris-Cl pH 7.5 used as the buffer (as used previously by R. Thatcher) and for the β-carboxymuconolactone hydrolase, 50 mM HEPES pH 8.6, containing 0.2 mM DTT and 10 % glycerol used as the buffer in all steps of the purifications.

2.6.2 DEAE anion exchange chromatography

The DEAE column (2.6 cm x 25 cm) was equilibrated with 1 l of 50 mM HEPES pH 7.5 and crude extract was loaded onto it. The column was washed with 0.7 l of the same buffer. The proteins were eluted using a 700 ml NaCl gradient (0 M – 0.5 M NaCl) using a flow rate of 2.5 ml/min and collecting 7 ml fractions. SDS-PAGE was carried out to find the fractions with the target protein. Enzyme activity assays were performed on these fractions. Those with highest activity were pooled together. The combined fractions from the DEAE column were concentrated by using a Pierce™ Protein Concentrator PES, 10K MWCO and centrifuging at 4000 x g to a final volume of 10 ml.

2.6.3 Hydrophobic reaction chromatography

The combined fractions from the DEAE column were concentrated using a Pierce™ Protein Concentrator PES, 10K MWCO and centrifugation at 4000 x g to a final volume of 11 ml. Ammonium sulfate was added to 20% saturation. Precipitated proteins were removed by centrifugation at 5000 x g for 15 min. The supernatant was loaded on a phenyl-Sepharose column (12 cm x 2 cm) equilibrated with HEPES 50 mM, glycerol 10%, DTT 0.2mM, pH 8.4, containing 20% saturated ammonium sulfate. Elution was done using a linear gradient (700 ml) from 20% - 0% saturated ammonium sulfate in the same buffer and a flow rate of 2 ml/min. To identify the fractions containing the target protein, SDS-PAGE was carried out along with enzyme activity assays, and the fractions with target protein were pooled together. This step used only for the β-carboxymuconolactone hydrolase.

2.6.4 Gel filtration Chromatography

The pooled and concentrated fractions from the DEAE step were loaded on the S-300 gel filtration chromatography column (50 cm x 2 cm) equilibrated with 1l of 50 mM HEPES pH 7.5, containing 0.2 M NaCl. The same buffer (0.7 l) was used to elute the enzyme using a flow rate of 1 ml/min and collecting 5 ml fractions. To identify the fractions containing the target protein, SDS-PAGE was carried out along with enzyme activity assays, and the fractions with the target protein were pooled together and concentrated using a Pierce™ Protein Concentrator PES, 10K MWCO and centrifuging at 4000 x g.
The purified protein was stored in aliquots at -80 °C until use.
In case of β-carboxymuconolactone hydrolase, the pooled and concentrated fractions from the phenyl-Sepharose column were loaded on the S-300 gel filtration chromatography column.

2.7 Assays for enzymes in the catechol branch of the β-ketoadipate pathway

The assays for enzymes in downstream reactions of the catechol branch were carried out at 25°C using a Cary Bio50 spectrophotometer and 50 mM HEPES, pH 7.5 as the assay buffer, in a total volume of 1 ml. Muconolactone isomerase activity was assayed by the disappearance of muconolactone[20], as monitored at 230 nm (ε = 1428 $M^{-1}cm^{-1}$), in the presence of β-ketoadipate enol-lactone hydrolase. As described by L. N. Ornston[14], muconolactone isomerase shifts the endocyclic double bond in *cis,cis*-muconolactone (substrate) and produces β-ketoadipate enol-lactone (product). However, these compounds absorb equally at 230 nm. On the other hand, the product of β-ketoadipate enol-lactone hydrolase, β-ketoadipate, does not absorb at 230 nm. Therefore, the isomerase activity can be measured by the decrease in absorbance at 230 nm in the presence of β-ketoadipate enol-lactone hydrolase. These assays were not done under conditions that allowed the individual activities to be measured so the results for these enzymes are only qualitative.

2.7.1 Production of muconolactone from *cis,cis*-muconate

Muconolactone was prepared from *cis,cis*-muconate (ε_{260} =16,900 M^{-1}cm-1) in the presence of partially purified muconate cycloisomerase from *Pseudomonas putida* strain PB2701. Reaction mixtures (1 ml) were prepared in 50 mM HEPES, pH 7.5, 2 µM $MnCl_2$ and various amounts of *cis,cis*-muconate (Sigma). The final concentration of muconolactone was estimated using the extinction coefficient 1428 $M^{-1}cm^{-1}$ at 230 nm and assuming full conversion.

2.8.1.1 Assay of muconolactone isomerase: sample calculation

ε of muconolactone[14, 20]= 1428 $M^{-1}cm^{-1}$ at 230 nm

Rate of Muconolactone isomerase Gel filtration pool = 0.2037 AU/min

Assay volume = 1213 µl

Dilution factor = 1

Enzyme volume added to the assay = 3 µl

Muconolactone isomerase activity = Assay volume*Rate/ l*ε

= 0.2037/1428*1.213

= (0.173 µmol/min)

Muconolactone isomerase protein content (mg) = Enzyme concentration * volume used in the assay

Standard curve equation: y=0.0097x+0.1273

Total volume of BCA assay= 225µl

Volume of enzyme added to BCA assay= 25 µl

Volume of enzyme added to activity assay= 10 µl

= [(0.4469 -0.1273)/0.0097)*(225/25)] * 10/1000

= 0.002965361 (mg)

Undiluted Muconolactone isomerase specific activity = Activity / protein content (mg)

= 0.173/0.002965361

= 58.35 (umol/min/mg)

2.7.2 Production and purification of muconate cycloisomerase from *Pseudomonas putida* strain PB2701:

Attempts to clone and express the gene (*catB*) encoding muconate cycloisomerase from *P. putida* were not successful (data not shown). Therefore, a source of the enzyme was obtained by culturing *P. putida* PB2701 at 30 ^{O}C in basal salt medium (BSM). This medium was prepared by dissolving 25.3 g K_2HPO_4 and 22.5 g of NaH_2PO_4, in 1 l deionized water added to a combination of 5 g $(NH_4)SO_4$ and 2 g $MgSO_4{\cdot}(H_2O)_7$ dissolved in 1 l deionized water.

Induction of catabolic enzyme activities of *P. putida* PB2701 was performed by growth for 24 hours in the BSM medium supplemented with 10 mM benzoic acid, as Ornston reported[20]. After 5 h 10 ml additional benzoic acid 0.5 M added and growth was continued for 3 h. Cells were harvested by centrifugation at 7500 x g at 4 °C for 20 min and the pellet was resuspended in disodium potassium phosphate buffer 20 mM, pH 6.8. The cell suspension was sonicated and centrifuged as described above: the supernatant (crude extract) was separated from the pellet and ammonium sulfate was added to 20% saturation. Precipitated proteins were removed by centrifugation at 6000 x g for 20 min. The supernatant was loaded on a phenyl-Sepharose column (12 cm x 2 cm) equilibrated with 20 mM disodium potassium phosphate buffer, pH 6.8, containing 20% saturated ammonium sulfate. Elution was done using a linear gradient (700 ml) from 20% - 0% saturated ammonium sulfate in the same buffer and at a flow rate of 2 ml/min. To identify the fractions containing the target protein enzyme activity assays carried out by measuring the disappearance of absorbance at 260 nm of *cis,cis*-muconate, and the fractions with target protein were pooled together.

2.8 Assays for enzymes in the protocatechuate branch of β-ketoadipate pathway

For assays of β-carboxymuconolactone hydrolase/decarboxylase the substrate β-carboxymuconolactone was produced *in situ* using purified protocatechuate 3,4-dioxygenase from *A. niger* (gift of P. Semana) and partially purified β-carboxymuconate cyclase (see section 2.7.1). A mixture of 50 mM Tris-Cl buffer, pH 7.5, including 40 µl 0.01 mM 3,4-dihydroxybenzoic acid and 5 µl 2 mg/ml protocatechuate 3,4-dioxygenase in a total volume of 1 ml was prepared in order to produce β-carboxymuconolactone. To confirm the synthesis of this metabolite the decrease in absorbance of protocatechuate at 290 nm was monitored.

After completion of the reaction, 5 µl of 0.57 mg/ml of β-carboxymuconate cyclase (partially purified) was added to the mixture and the reaction was monitored by the decrease in absorbance at 260 nm,

following formation of the β-carboxymuconolactone[13]. After completion of the reaction 5 µl of 8.3 mg/ml β-carboxymuconolactone hydrolase/decarboxylase was added and the changes in absorbance monitored at 230 to follow conversion of β-carboxymuconolactone to β-ketoadipate[13].

Sample calculations are shown below. In this calculation the extinction coefficient at 230 nm of β-carboxymuconolactone[18, 23] used was 8500 M^{-1} cm^{-1}.

Sample calculation:

(Rate) dA_{230}/min = l*ε*(dc/min); where l = 1cm

ε of β-carboxymuconolactone = 8500 $M^{-1}cm^{-1}$

λ_{max}=230

Rate of β-carboxymuconolactone hydrolase/decarboxylase gel filtration pool = 0.289

Assay volume = 1005 µl

Enzyme volume added to the assay = 5 µl of 1:10 gel filtration sample

β-carboxymuconolactone hydrolase/decarboxylase activity = Assay volume*Rate/ l*ε

= 0.289 AU/8500 $M^{-1}cm^{-1}$ min^{-1}= 0.000034 mol/l min^{-1}

= 0.000034 mol/l min^{-1} * 0.001005 l = 0.00000003417 mol min^{-1}

= 0.00000003417 mol min^{-1} * 1005/5 (dilution factor)

= 0.000006868 mol min^{-1}

= 6.87 µmol/min in 1:10 enzyme sample enzyme sample

Therefore, 68.7 µmol/min in undiluted gel filtration enzyme sample (1 ml)

Therefore 824.4 µmol/min total activity in 12 ml.

β-carboxymuconolactone hydrolase/decarboxylase protein content (mg) = Enzyme concentration * volume used in the assay

Standard curve equation: y=0.0092x+0.2109

Total volume of BCA assay= 225 µl

Volume of enzyme added to BCA assay= 25 µl of a 1:10 dilution

Dilution factor = 10

β-carboxymuconolactone hydrolase/decarboxylase protein concentration = [((1.064 - 0.2109)/0.0092))*(225/25)] * 10

= 8345 µg/ml undiluted gel filtration sample

In total, there is 8345 µg/ml * 12 ml = 100.14 mg

Undiluted specific activity = Activity / protein content

= 824.4 µmol/min / 100.14 mg

= 8.23 µmol/min/mg

2.8.1 Determination of β-carboxymuconate cyclase profile:

The pH-activity optimum was determined by carrying out assays in MOPS, MES, Tris-HCl, HEPES, and sodium acetate buffers within the pH range of 4-9 and concentrations of 50 mM.

2.8.2 Monitoring spectral changes for reactions of the protocatechuate branch of the β-ketoadipate pathway

As described by Cain *et al.* characteristic spectral changes are observed for the conversion of protocatechuate to β-ketoadipate[18]. A brief description of the procedure is as follows.

First, the UV spectrum of 50 mM Tris-HCl buffer pH 7.5 was recorded as a baseline in a 10 mm quartz cuvette. The spectrum of 50 mM Tris-HCl buffer pH 7.5 (1 ml) plus 40 µl of protocatechuate 0.01M was recorded over the range of 200 to 350 nm. Purified protocatechuate 3,4-dioxygenase (0.01 mg) was added to the assay solution and the spectral changes were recorded. Every 5 min protocatechuate 3,4-dioxygenase was added to the reaction mixture in the cuvette. After spectral changes ceased, 0.28 mg β-carboxymuconate cyclase was added to the assay solution and the resulting spectral changes were recorded. The enzyme was added to the solution every 5 min until no additional changes were observed in the spectrum. At this point, 0.041 mg β-carboxymuconolactone hydrolase added to the cuvette and

spectral changes were recorded. Enzyme was added until of no additional changes in the spectrum were observed.

2.9 Protein analysis by SDS-PAGE and BCA assay

To estimate the purity of the protein SDS-PAGE (12 % acrylamide gel) was used[51, 52]. Protein concentrations were estimated according to the instructions from the manufacturer of the Pierce BCA Protein assay kit from Thermo Fisher Scientific using bovine serum albumin for the standard curve at 34 ºC (Figure 2.1).

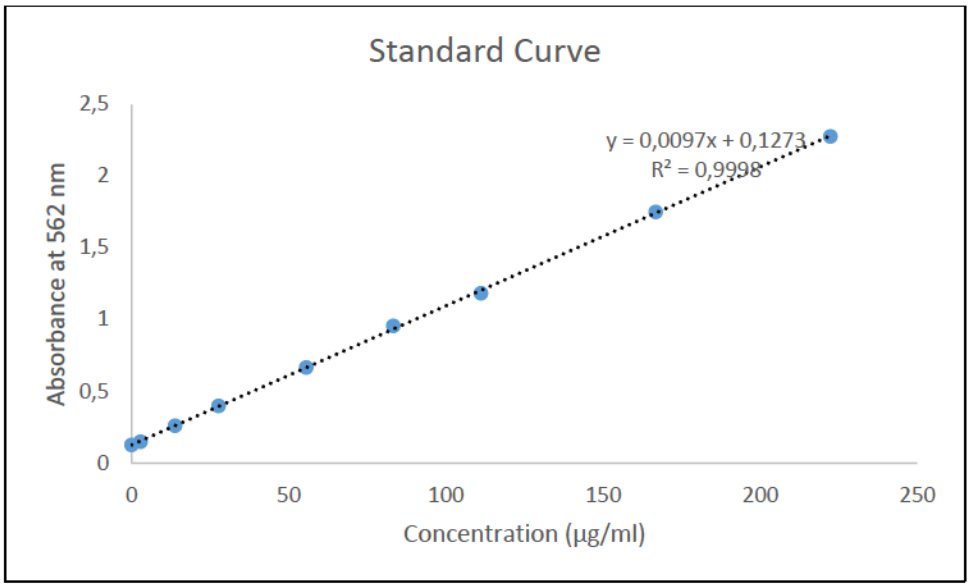


Figure 2.1: Bovine serum albumin standard curve generated based on the Pierce BCA Protein assay.

2.10 Techniques for examining the structural properties of the purified enzymes

2.10.1 Analytical ultracentrifugation (AUC)

All the samples were diluted to absorbance values between 0.7 and 1.0 at 280 nm. The titanium cuvettes had a path length of 12 mm. Sedimentation velocity experiments were carried out on a Beckman Optima XL-I ultracentrifuge using the An-60 Ti rotor at 4°C. The samples were centrifuged at 35,000 rpm, and 200 radial absorbance scans of the sample against the buffer were collected at a wavelength of 280 nm. Buffer density and viscosity were calculated using Sednterp 2.0[53, 54] according to the given composition: partial specific volume of the protein samples used was 0.73 cm^3/g and the buffer density and viscosity used was 1.0021 g/cm^3 and 1.03 s/cm^2. Sedimentation coefficient

distributions, g(s*), and molecular weights were analyzed by the standard c(s) model in DCDT[55]+version 14.4.

2.10.2 Size exclusion chromatography (SEC) to estimate molecular weight

A Superdex-75 10/300 GL (GE LifeSciences) gel filtration column was used to determine the oligomeric state of each purified protein and estimate their molecular weights (MWs). The column, connected to a BioRad DuoFlow System, had a flow rate of 0.5 mL/min after 1 ml of protein solution was injected; absorbance at 280 nm was collected. The running buffer used was 50 mM Tris-HCl pH 7.4 containing 100 mM NaCl. Gel filtration standards (Bio-Rad, catalog # 1511901) were used to calibrate the column and generate a standard curve for estimating MWs: Blue Dextran 2000 (Amersham Biosciences) was used to estimate the void volume of the column.

3 Results

3.1 Genes identified by querying *A. niger* genome with genes from *A. nidulans*

Genome sequences of *Aspergillus niger* are available from the Joint Genome Institute. Sequences of putative β-ketoadipate pathway genes in *Aspergillus nidulans*, based on the studies by T.M. Martins *et al.*[21] (Table 3.1) were used in BLAST searches against the *Aspergillus niger* genome using two databases:

a. JGI http://genome.jgi.doe.gov/Aspni7/Aspni7.home.html

b. NRRL3 https://mycocosm.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html[56]

Gene Code	Protein Assigned name	Pathway
AN1151	carboxy-cis,cis-muconate cyclase	Benzoate degradation and protocatechuate branch of the 3-ketoadipate pathway
AN5232	3-carboxymuconolactone hydrolase (decarboxylating)	
AN3895	muconate isomerase (CoA transferase family III)	Salicylate degradation and catechol branch of the 3-ketoadipate pathway
AN4061	muconolactone isomerase	
AN4531	3-ketoadipate enol-lactone hydrolase	

Table 3.1: Assigned genes in the 3-ketoadipate pathway in *Aspergillus nidulans*; adapted from: T.M. Martins et al[21].

Based on these Blast comparisons, five corresponding sequences in the *A. niger* genome were identified. Table 3.2 summarizes the best hits from the *A. niger* NRRL3 database for each of the predicted *A. nidulans* sequences.

3.2 Genes identified by querying *A. niger* genome with genes from *P. putida*

The gene, *catB*, from *Pseudomonas putida* was previously sequenced and identified as encoding muconate cycloisomerase 1 (MLEI) by Teri. et al[57]. By using this sequence as a query sequence in a BLAST search against the *A. niger* genome, NRRL3_11120 was identified as a gene possibly encoding

MLEI activity (Table 3.2). The BLAST comparison indicated sequence motifs for enolase-like, N-terminal (IPR029017), N-terminal domain (IPR013341), enolase C-terminal domain-like (IPR029065) and mandelate racemase/muconate lactonizing enzyme, C-terminal (IPR013342). However, the low e-value of 9e-22 and the sequence identity of only 29.38% are weak indicators that this protein has cycloisomerase activity.

3.3 Genes identified by querying *A. niger* genome with genes from *Acinetobacter calcoaceticus*

Two isozymes of β-ketoadipate enol-hydrolase are elaborated by *Acinetobacter calcoaceticus* in response to either protocatechuate (enol-lactone hydrolase I) or *cis,cis*-muconate (enol-lactone hydrolase II)[58]. A single enol-lactone hydrolase is induced by β-ketoadipate in *Pseudomonas putida*[59] . The sequence of the gene coding for β-ketoadipate enol-lactone hydrolase II from *Acinetobacter calcoaceticus*, was used to BLAST against the *A. niger* genome, and NRRL3_01409 was identified as a gene possibly encoding β-ketoadipate enol-lactone hydrolase activity (Table 3.2).

3.4 PCR amplification and plasmid construction

The targeted *A. niger* genes with the predicted enzyme activities as well as the expected lengths in base pairs are shown in Table 3.2.

Gene	Possible activities in β-ketoadipate pathway	Query Sequence	E value	Identity %	Coverage %	Base pair
NRRL3_02586	β-carboxymuconate cyclase	*A nidulans* AN1151	0.00E+00	91.39	100	1083
NRRL3_00837	β-carboxymuconolactone hydrolase	*A nidulans* AN5232	1.00 E-115	72.32	95	699
NRRL3_08340	β-carboxymuconolactone hydrolase	*A nidulans* AN5232	8.00E-100	33.61	85.92	429
NRRL3_10507	muconolactone isomerase	*A nidulans* AN4061	2.00E-61	68.83	77	447
NRRL3_07569	muconolactone isomerase	*A nidulans* AN4061	6.00E-18	32.76	65	921

Gene	Possible activities in β-ketoadipate pathway	Query Sequence	E value	Identity %	Coverage %	Base pair
NRRL3_01886	β-ketoadipate succinyl CoA transferase	*A nidulans* AN3895	0.00E-00	56.24	96.43	1515
NRRL3_01593	β-ketoadipate succinyl CoA transferase	*A nidulans* AN3895	1E-155	49.71	92	1644
NRRL3_11640	β-ketoadipate succinyl CoA transferase	*A nidulans* AN3895	7.00E-180	58.66	94	1557
NRRL3_01409	β-ketoadipate enol-lactonase 2	*A baylii* 3-oxoadipate enol-lactonase II	3.91E-019	34.7	26.9	1641
NRRL3_04788	β-ketoadipate enol lactone hydrolase	*A nidulans* AN4531	1.00E-123	63.12	48	792
NRRL3_11120	muconate cyclase	*P putida* P08310 *catB*	2.50E-013	38.1	36.1	1113

Table 3.2: Putative *A. niger* genes comparisons to genes from *A. nidulans*, *Acinetobacter calcoaceticus* and *P.putida*

PCR amplification was carried out as described in *Materials and Methods*, and PCR products were purified and inserted into the pLATE-11 vector using the ligation independent aLICator LIC Cloning kit: recombinant fragments (amplified inserts using plasmid primers) were detected with the expected size (Figure 3.1).

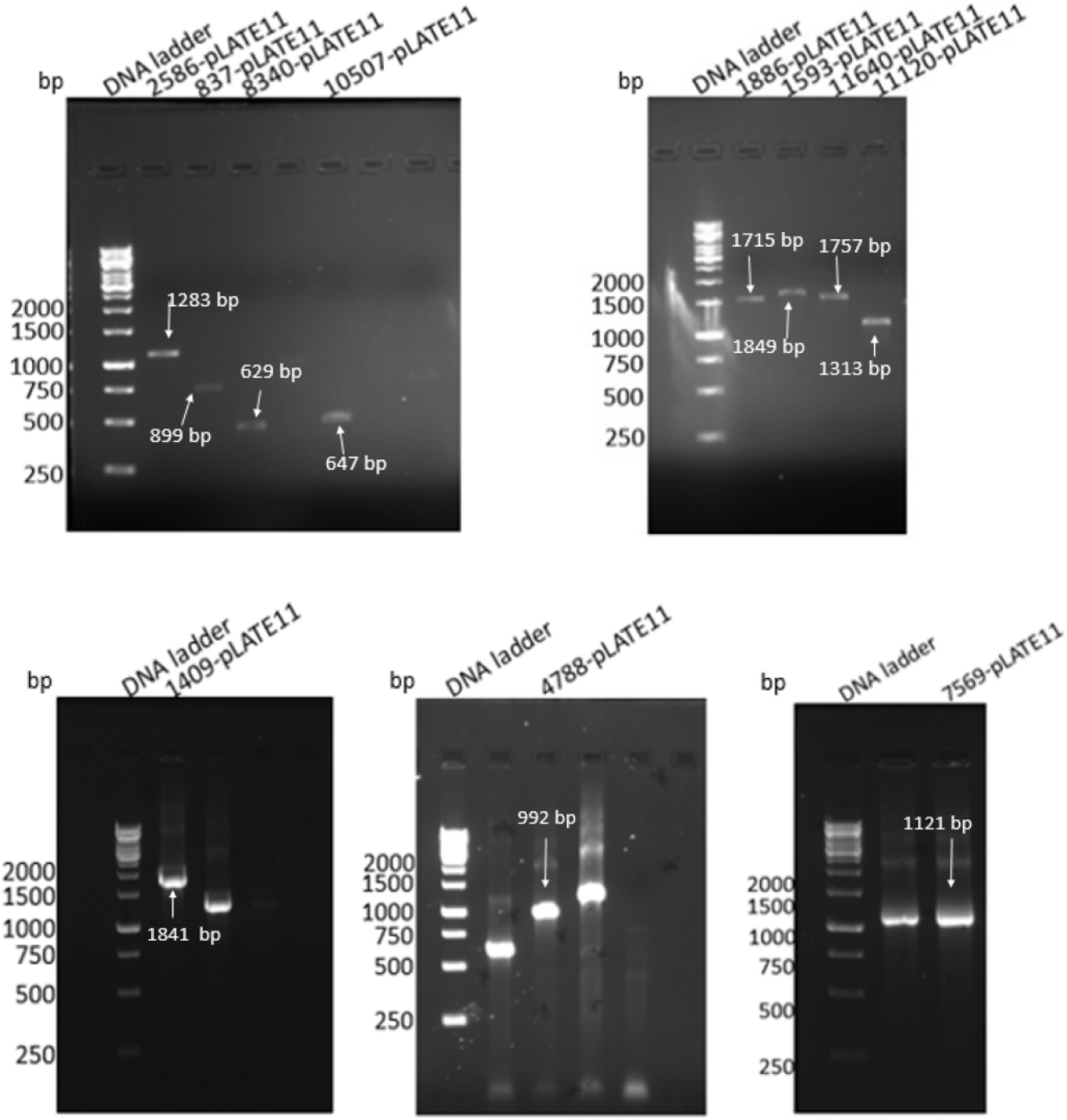


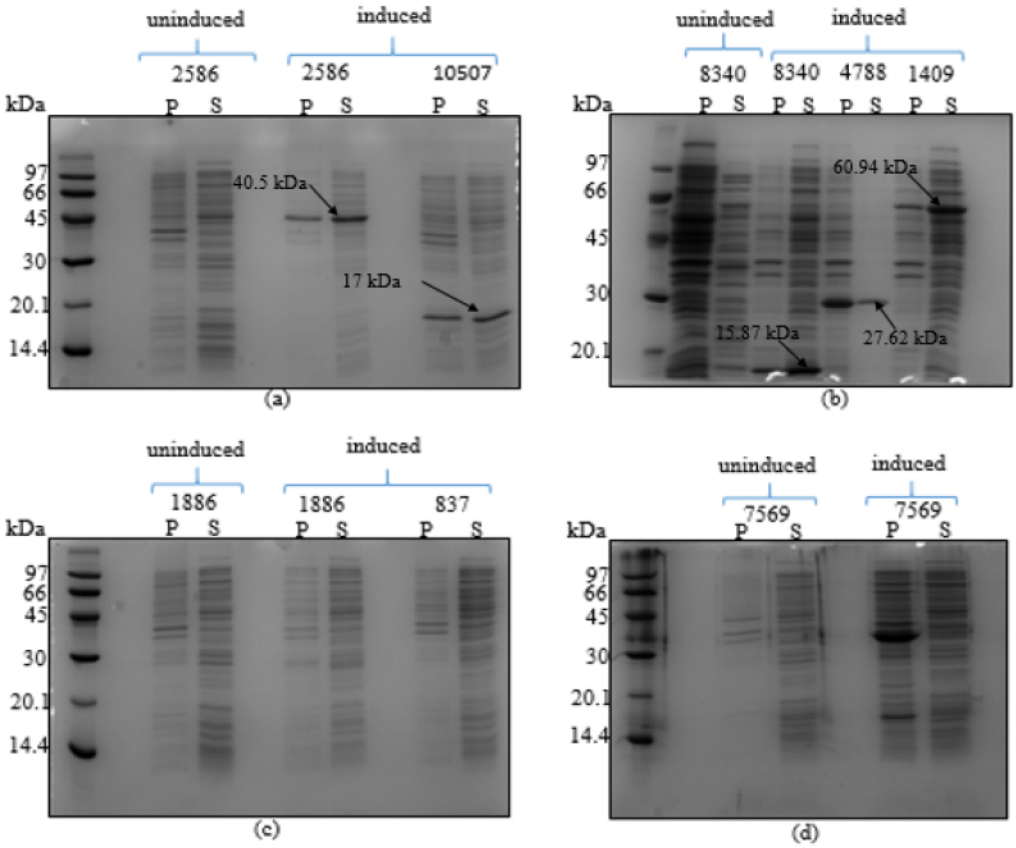
Figure 3.1: Genes of interest amplified with plasmid primers and separated by agarose gel as described in *Materials and Methods*

3.5 DNA sequencing

All the recombinant plasmids were sequenced using primers designed based on pLATE-11 vector sequence from the aLICator LIC cloning kit, essentially as described by the manufacturer[44]. The sequences of the plasmid inserts agreed with the sequence from the NRRL3 database (data not shown).

3.6 Expression of protein from recombinant plasmids

Recombinant plasmids were transformed into *E. coli* BL21 (DE3), grown in liquid medium and expression was induced as described in *Materials and Methods*. A non-induced culture was included as a control. SDS-PAGE gels show the expression levels of proteins in the pellet and supernatant fractions (Figure 3.2). The arrows point to proteins expressed in induced vs uninduced cells close to the predicted molecular weights of the proteins from the genes of interest. The arrows are labelled with the predicted molecular weights.



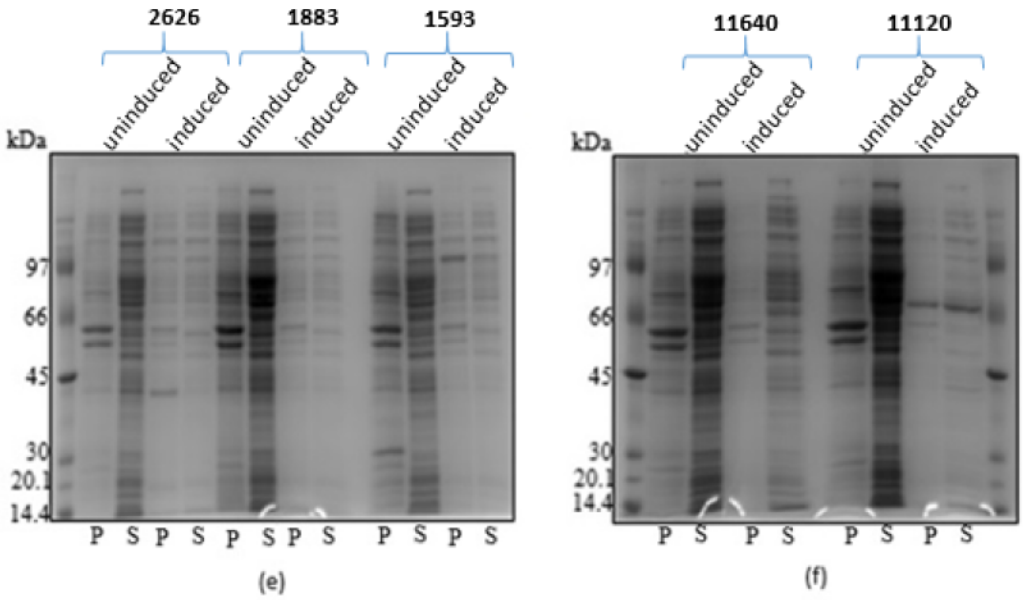


Figure 3.2: SDS-polyacrylamide gel (12%) electrophoresis of gene expression tests (a) NRRL3_02586, NRRL3_10507; (b) NRRL3_08340, NRRL3_04788, NRRL3_01409; (c) NRRL3_01886, NRRL3_00837; (d) NRRL3_07569; (e) NRRL3_02626, NRRL3_01883, NRRL3_01593; (f) NRRL3_11640, NRRL3_11120.

The results of these expression tests are summarized in Table 3.3. If a protein near the predicted molecular weight was detected in the induced v. uninduced sample supernatant, it was scored as "soluble"; if it was in the induced vs uninduced pellet it was scored as "insoluble"; and if no obvious induced band was detected, it was scored as "unsuccessful". By these criteria, five of the targeted genes were successfully expressed as soluble proteins.

Gene	Possible enzyme activities in β-ketoadipate pathway	Predicted molecular weights (kDa)	Expressed in *E.coli* BL21(DE3)
NRRL3_02586	β-carboxymuconate cyclase	40.5	soluble
NRRL3_00837	β-carboxymuconolactone hydrolase	25.5	unsuccessful
NRRL3_08340	β-carboxymuconolactone hydrolase	15.89	soluble
NRRL3_10507	muconolactone isomerase	17	soluble
NRRL3_07569	muconolactone isomerase	35.23	insoluble
NRRL3_01886	β-ketoadipate succinyl CoA transferase	54.47	unsuccessful
NRRL3_01593	β-ketoadipate succinyl CoA transferase	58.29	unsuccessful
NRRL3_11640	β-ketoadipate succinyl CoA transferase	55.51	unsuccessful
NRRL3_01409	β-ketoadipate enol lactone hydrolase	60.94	soluble
NRRL3_04788	β-ketoadipate enol lactone hydrolase	27.62	soluble
NRRL3_11120	muconate cyclase	39.69	soluble
NRRL3_01883	β-carboxymuconate cyclase	52.3	unsuccessful

Table 3.3: Results of recombinant expression in *E. coli* of enzymes encoded by the indicated genes

3.7 Enzymatic assays:

3.7.1 Assay of expressed proteins for enzyme activities of the catechol branch of the β-ketoadipate pathway

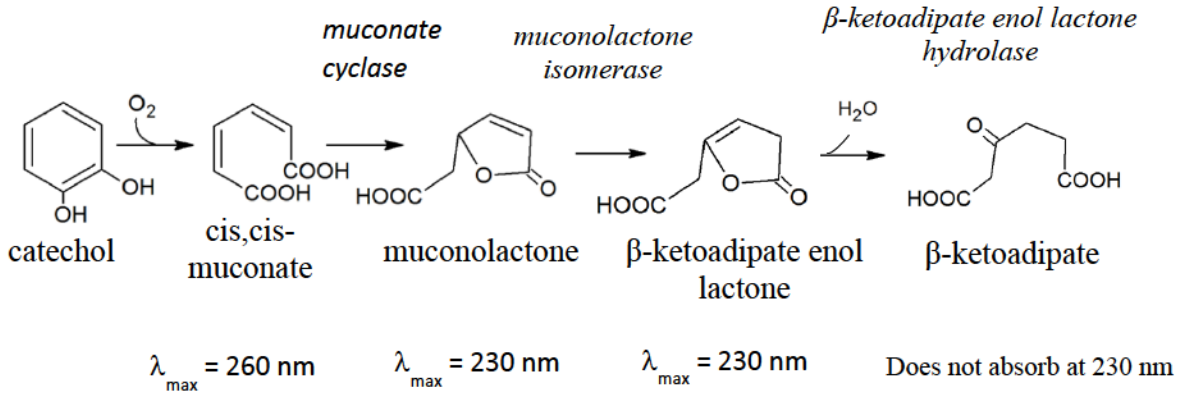


Figure 3.3: Intermediates in the catechol branch of the β-ketoadipate pathway together with enzyme names

The best candidate in the *A. niger* genome, identified by similarity with the known bacterial enzyme sequence for muconate cyclase is NRRL3_11120 (Table 3.2). An extract of *E. coli* expressing the corresponding protein was not active in assays for this activity (data not shown). On the other hand, a partially purified preparation of muconate cyclase from *Pseudomonas* (see *Materials and Methods*) was active, generating a product from *cis,cis*-muconate with absorbance at 260 nm (Fig. 3.4A).

On the basis of sequence comparisons with *A. nidulans* proteins implicated in the catechol branch of the β-ketoadipate pathway[21] (Table 3.2), the most likely candidates to have muconolactone isomerase and β-ketoadipate enol lactone hydrolase activities are NRRL3_10507 and NRRL3_04788, respectively. As summarized in *Materials and Methods*, muconolactone and β-ketoadipate enol lactone absorb at 230 nm whereas β-ketoadipate does not absorb at this wavelength. Therefore, any changes in absorbance of a solution of muconolactone should correspond to the combined activity of these two enzymes. Consistent with this, the absorbance at 230 nm of an assay mixture containing muconolactone (Fig. 3.4A) did not change upon the addition of NRRL3_10507 (Fig. 3.4 B) and then decreased upon the addition of crude extract of NRRL3_04788 (Figure 3.4 C). This result is qualitatively similar to those originally reported for the activity assays of the corresponding enzymes from *Pseudomonas putida*[14], and is consistent with muconolactone isomerase activity and β-ketoadipate enol lactone hydrolase activity for NRRL3_10507

and NRRL3_04788 proteins respectively. However, there were no changes in absorbance at 230 nm with the addition of crude extract of NRRL3_01409 instead of NRRL3_04788, another candidate for β-ketoadipate enol lactone hydrolase activity (data not shown).

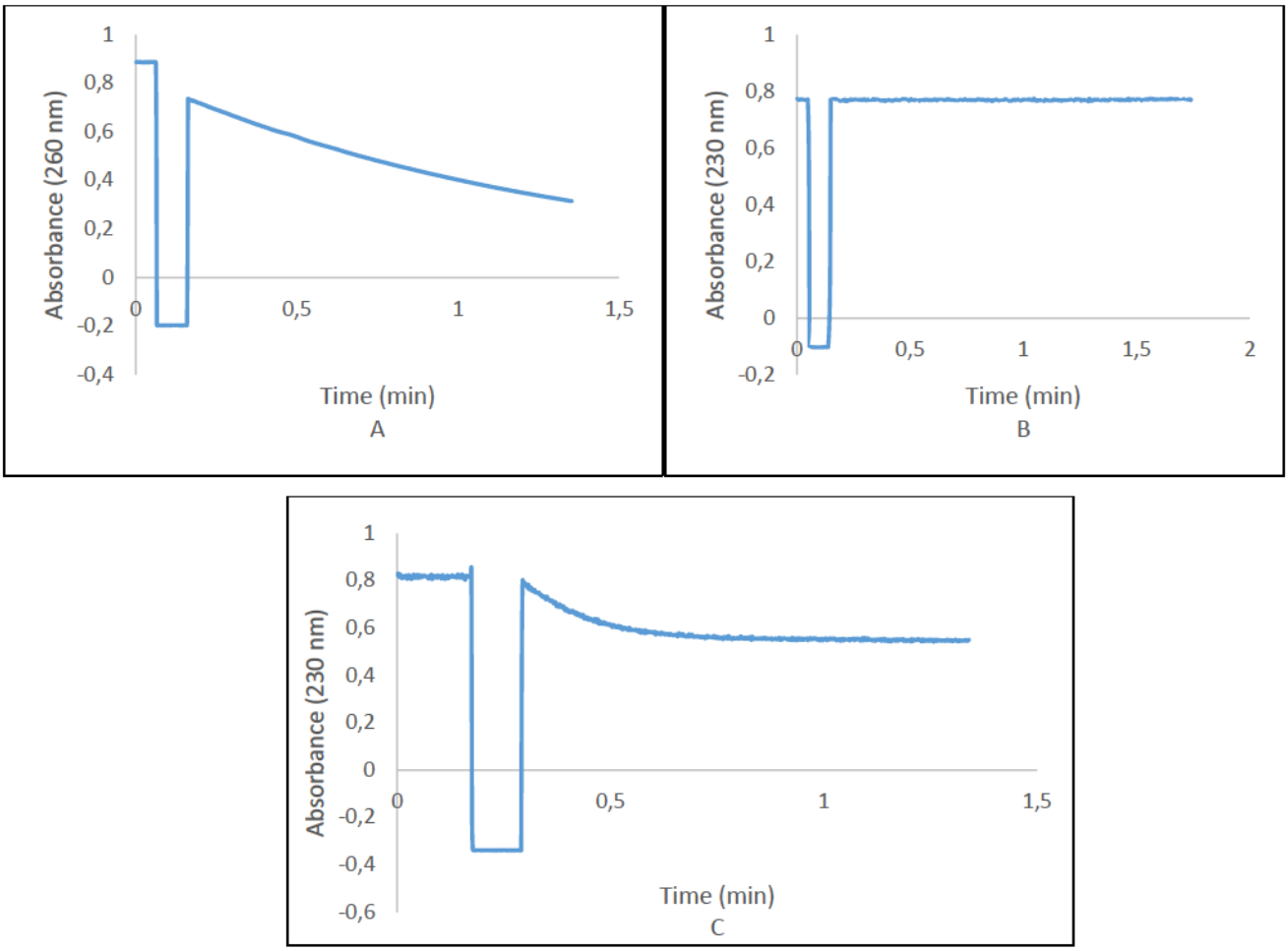
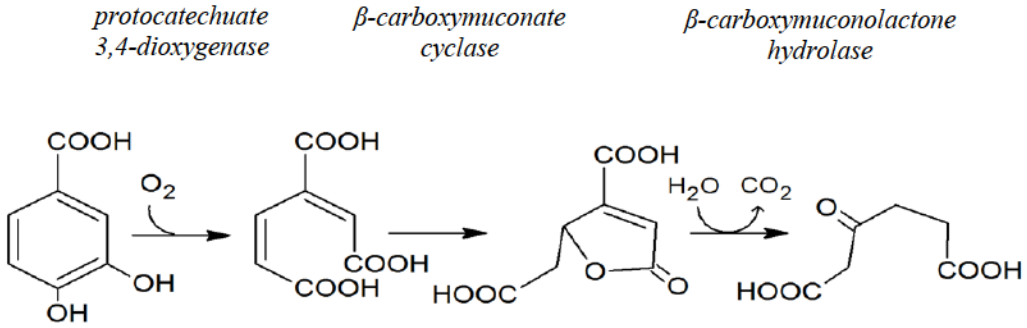


Figure 3.4: Assays of enzymes of the catechol branch of the β-ketoadipate pathway.

A. Change in absorbance at 260 nm of a *cis,cis*-muconate-containing assay mixture upon the addition of partially-purified muconate cycloisomerase from *Pseudomonas*. The assay (1 ml) contained 50 mM HEPES, pH 7.5, 53 µM *cis,cis*-muconate, 2 µM $MnCl_2$ and 0.57 mg/ml partially purified muconate cycloisomerase; **B**. Change in absorbance at 230 nm upon addition of an *E. coli* extract expressing NRRL3_10507 to an assay mixture containing muconolactone (approx.0.57 mM); **C**. Change in absorbance at 230 nm upon addition of an *E. coli* extract expressing NRRL3_04788 to the assay mixture shown in **B**. Assay procedures were as described in *Materials and Methods*. The absorbance drops in

each continuous trace are due to removal and reinsertion of the assay cuvette for the additions of enzyme and mixing.

3.7.2 Assay of expressed proteins for enzyme activities of the protocatechuate branch of the β-ketoadipate pathway



λ_{max} = 290 nm	λ_{max} = 260 nm	λ_{max} = 230 nm	Does not absorb at 230 nm
protocatechuic acid	β-carboxy-cis,cis-muconate	β-carboxymucono-lactone	β-ketoadipate

Figure 3.5: Intermediates in the protocatechuate branch of the β-ketoadipate pathway

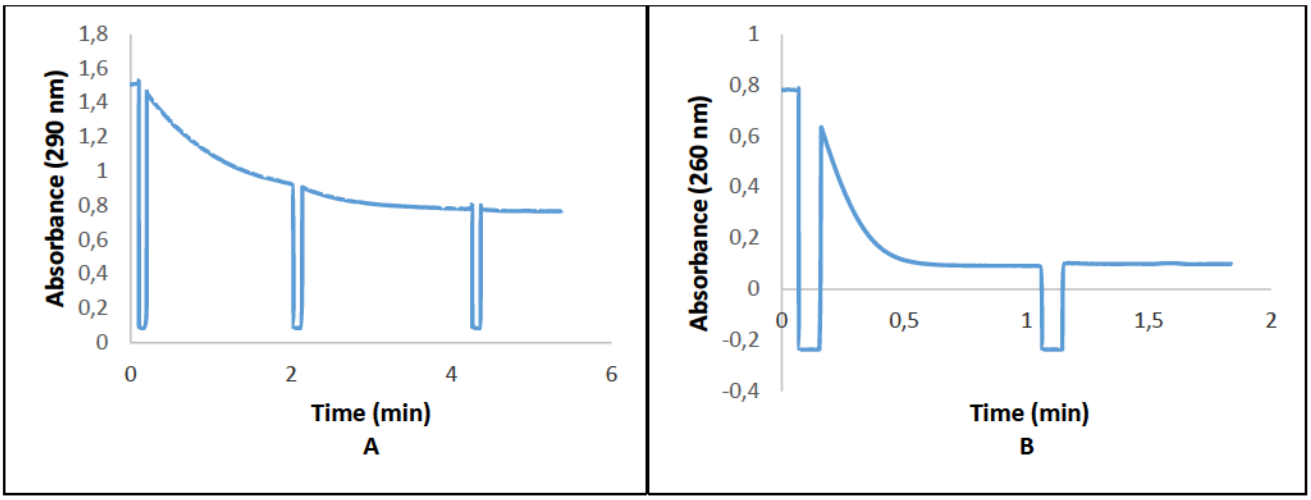
The first enzyme in the pathway in *A. niger* was identified and expressed by Patrick Semana in our laboratory[60]. Addition of the purified dioxygenase to a solution of protocatechuate resulted, as expected, in a decrease in absorbance at 290 nm (Fig 3.6A).

NRRL3_02586 is proposed to encode β-carboxymuconate cyclase activity on the basis of similarity with the *A. nidulans* orthologue, as well as with a characterized β-carboxymuconate cyclase from the fungus, *Neurospora crassa* (Table 3.2). Since the substrate, β-carboxy-*cis,cis*-muconate, has a strong absorption peak at 260 nm, addition of a crude extract of *E. coli* expressing NRRL3_02586 to the assay solution should result in a decrease in absorbance at 260 nm related to the consumption of β-carboxy-*cis,cis*-muconate This was observed (Figure 3.6B), indicating β-carboxymuconate cyclase activity of the NRRL3_02586-encoded protein. These results are consistent with the study that has been reported by R.Thatcher et al[23].

β-carboxymuconolactone, the product of the previous reaction, absorbs at 230 nm and its enzymatic conversion product, β-ketoadipate, does not, as reported by Cain *et al.*[18]. We thus expected to see a decrease in absorbance of a solution of β-carboxymuconolactone by adding crude extract of *E. coli* expressing NRRL3_08340, initially assigned as β-carboxymuconolactone hydrolase on the basis of

querying the *A. niger* NRRL3 genome with AN5232 from *A.nidulans.* However, there was no change in absorbance observed (data not shown).

R.Thatcher *et al.*[24] purified β-carboxymuconolactone hydrolase from *Aspergillus niger* and reported that the enzyme purified as a single polypeptide that has both decarboxylation and hydrolysis activity and a molecular weight of 54 kDa. In contrast, the protein expressed from NRRL3_08340 has a molecular weight of almost 16 kDa, which is only about a third of the size of the enzyme characterized by Thatcher *et al.* On the other hand, NRRL3_01409 which has some sequence similarity to 3-oxoadipate enol-lactonase II from *Acinetobacter baylii* (Table 3.2) did not appear to have activity on β-ketoadipate enol lactone as the substrate in our experiments (data not shown). However, the molecular weight of this protein is 60 kDa, similar to the 54 kDa of the enzyme studied by Thatcher *et al.* The activity of NRRL3_01409 was therefore assayed using β-carboxymuconolactone as the substrate. Interestingly, a decrease in absorbance at 230 nm was observed (Figure 3.6 C). This indicates the β-carboxymuconolactone hydrolase activity of protein encoded by NRRL3_01409 rather than β-ketoadipate enol lactone hydrolase activity.



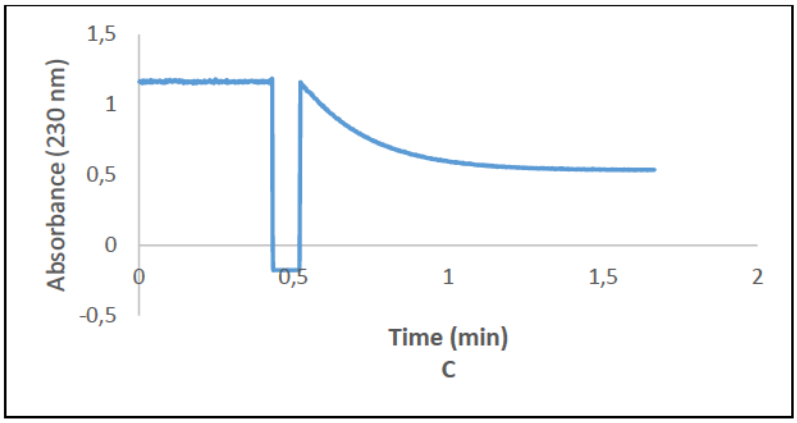


Figure 3.6: Assays of enzymes of the protocatechuate branch of the β-ketoadipate pathway

A. Changes in absorbance at 290 nm of a protocatechuate-containing assay mixture upon 3 successive additions of protocatechuate 3,4-dioxygenase; **B** changes in absorbance at 260 nm upon additions of 0.28 µg crude extract protein, twice, from cells expressing NRRL3_02586 to an assay mixture containing β-carboxymuconate; **C** changes in absorbance at 230 nm resulting from the addition of crude extract from cells expressing NRRL3_01409 to an assay mixture containing β-carboxymuconolactone. Assay procedures were as described in *Materials and Methods*. The absorbance drops in each continuous trace are due to removal and reinsertion of the assay cuvette for the additions of enzyme and mixing.

3.8 Purification of enzymes

3.8.1 Purification of muconoloactone isomerase encoded by NRRL3_10507

The protein encoding a putative muconoloactone isomerase (encoded by NRRL3_10507) was expressed in *E. coli* BL21(DE3) and purified as described in "Materials and Methods". Briefly, protein expression was induced by addition of IPTG and the cells were harvested by centrifugation. Cells were broken using sonication, and the enzyme was purified from the resulting crude extract by DEAE anion exchange chromatography followed by concentration and gel filtration chromatography. SDS-polyacrylamide gel electrophoresis of the gel filtration fractions, showed one major band of 17 kDa corresponding to the muconoloactone isomerase predicted molecular weight. The summary of muconoloactone isomerase purification is shown in Table 3.4 and Figure 3.7.

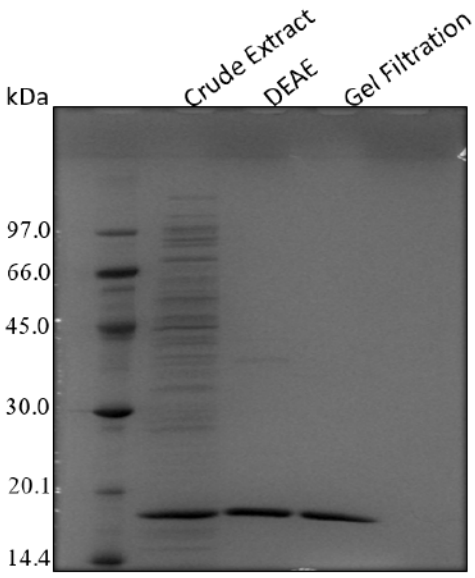


Figure 3.7: SDS-PAGE gel (12%) of the different steps of the purification of muconoloactone isomerase. In each well 25 µg of protein was loaded.

Step	Volume (ml)	Protein amount (mg)	Total activity (units)	Specific activity (µmol/min/mg)	Purification (fold)	Yield (%)
Crude extract	58	526	5003	9.5	1	100
DEAE	14.5	115	4549	39.4	4.1	90.9
Gel filtration	10	59	209	3.5	7.5	4.1

Table 3.4 Summary of the purification of muconoloactone isomerase

3.8.2 Purification of β-ketoadipate enol-lactone hydrolase

The protein encoding a putative β-ketoadipate enol-lactone hydrolase (encoded by NRRL3_04788) was expressed in *E. coli* BL21(DE3) and purified as described in "Materials and Methods". Briefly, protein expression was induced by addition of IPTG, the cells were harvested, broken by sonication and centrifuged in order to obtain the crude extract. The soluble protein was purified by using DEAE anion exchange chromatography, concentrated, and then further purified by gel filtration chromatography. Fractions from the gel filtration step were run on SDS-polyacrylamide gel electrophoresis, which showed one major band of 27.62 kDa representing β-ketoadipate enol-lactone hydrolase predicted molecular weight. Table 3.5 and Figure 3.8 summarize the β-ketoadipate enol-lactone hydrolase purification.

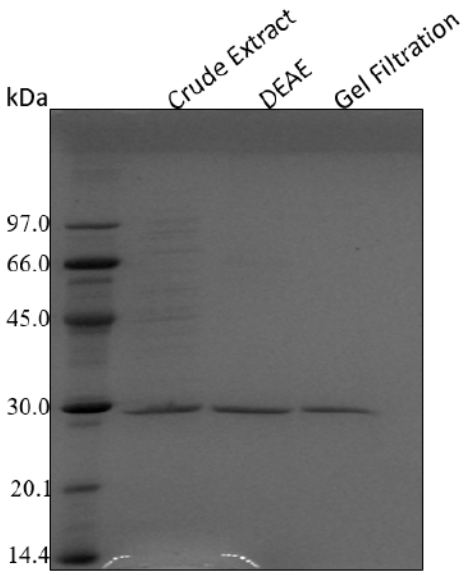


Figure 3.8: SDS-PAGE gel (12%) showing the purification steps of β-ketoadipate enol-lactone hydrolase. In each well 25 µg of protein was loaded.

Step	Volume (ml)	Protein amount (mg)	Total activity (units)	Specific activity (µmol/min/mg])	Purification (fold)	Yield (%)
Crude extract	50	1150	7189	6.24	1	100
DEAE	22	355	2300	6.46	1.03	31.99
Gel filtration	10	115	699	6.08	0.95	9.72

Table 3.5 Summary of the purification of β-ketoadipate enol-lactone hydrolase

3.8.3 Purification of β-carboxymuconate cyclase

The protein encoding a putative β-carboxymuconate cyclase (encoded by NRRL3_02586) was expressed in *E. coli* BL21(DE3) and partially purified as described in "Materials and Methods". Briefly, protein expression was induced by the addition of IPTG, the cells were harvested and then broken by sonication. The protein was partially purified from the resulting crude extract by DEAE anion exchange chromatography. An SDS-PAGE gel of fractions from the DEAE column is shown in Figure 3.9.

The pH-activity profile obtained with the purified enzyme reveals that the enzyme has the highest activity of 0.48 U/mg at pH 6.5 (Figure 3.10). This result is consistent with the results reported by Thatcher *et al.*[23], who reported that the pH optimum for a β-carboxymuconate cyclase purified from *A. niger* is at pH 6.

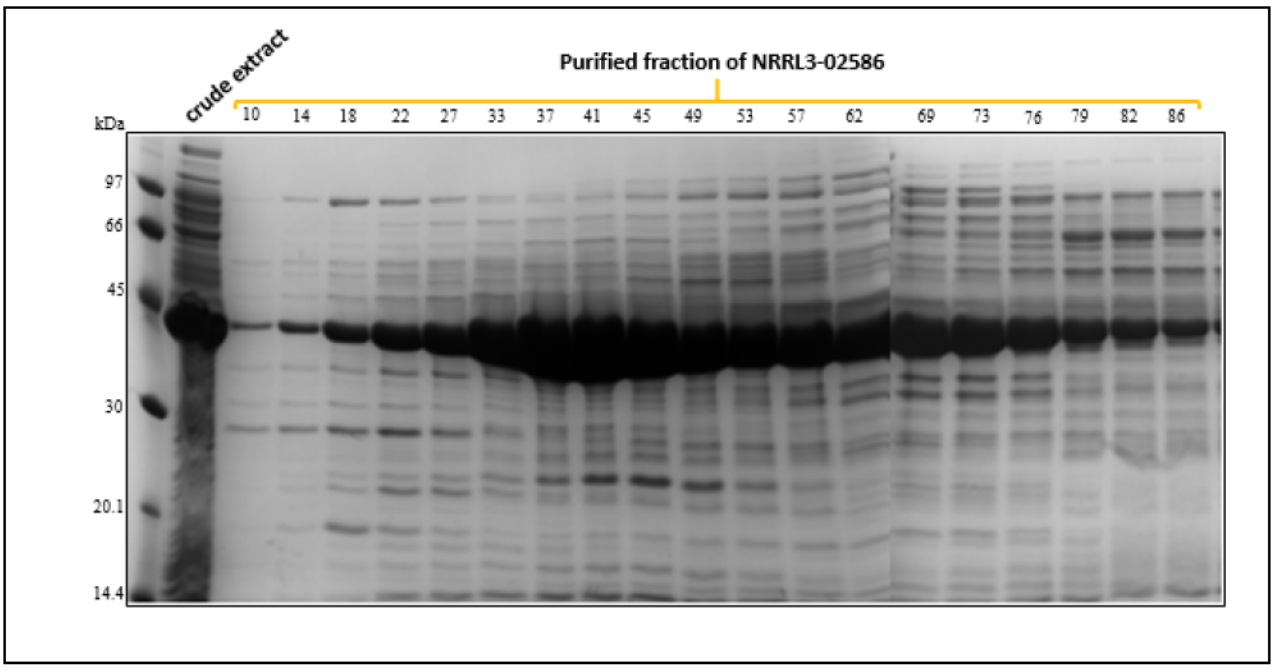


Figure 3.9: SDS-PAGE gel (12%) showing the β-carboxymuconate cyclase fractions purified by DEAE anion exchange chromatography.

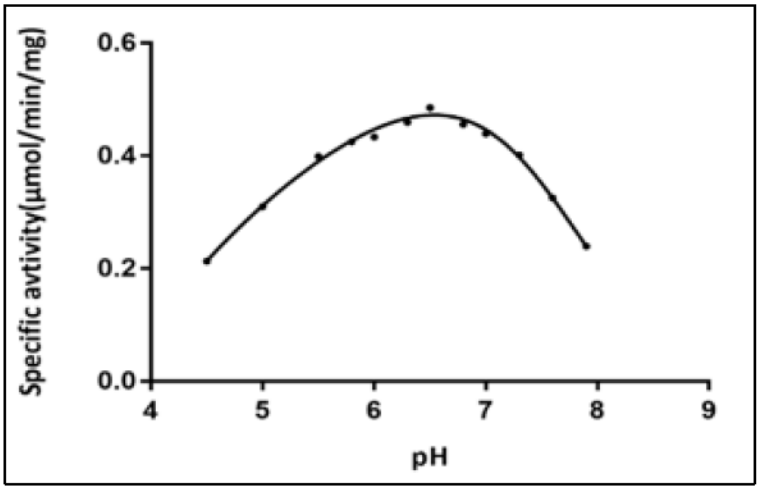


Figure 3.10: pH optimum for β-carboxymuconate cyclase at 25 °C using 50 mM MOPS or MES buffers at the indicated pH values, as described in Materials and Methods.

3.8.4 Purification of β-carboxymuconolactone hydrolase

The protein encoding a putative β-carboxymuconolactone hydrolase (encoded by NRRL3_01409) was expressed in *E. coli* BL21(DE3) and purified as described in "Materials and Methods". In brief, IPTG

was used to induce protein expression, cells were harvested and broken by sonication. The enzyme was purified by using DEAE anion exchange chromatography followed by phenyl-Sepharose chromatography and finally gel filtration chromatography, as described in *Materials and Methods*. The purification of β-carboxymuconolactone hydrolase is summarized in Table 3.6 and Figure 3.11. A specific activity of 8.3 U/mg was calculated, with 0.34-fold purification. The drop in specific activity in the gel filtration steps indicates some instability of the enzyme, as was observed by Thatcher and Cain[24]The source of this instability has not been determined.

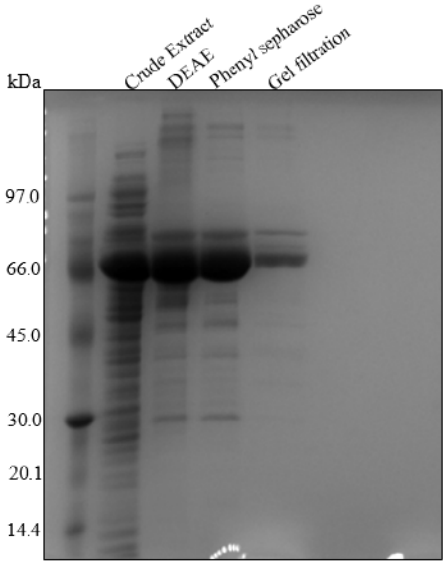


Figure 3.11: SDS-PAGE gel (12%) showing the purification of β-carboxymuconolactone hydrolase. In each well 25 µg of sample protein was loaded.

Step	Volume (ml)	Protein amount (mg)	Total activity (units)	Specific activity (µmol/min/mg)	Purification (fold)	Yield (%)
Crude extract	61	2910.8	$5.785*10^4$	19.9	1	100
DEAE	25	909.4	$2.198*10^4$	24.2	1.2	38
phenyl-Sepharose	17	594.3	$1.686*10^4$	28.4	1.4	29.14
Gel filtration	12	100	$0.827*10^3$	8.3	0.34	3.76

Table 3.6: Summary of purification of β-carboxymuconolactone hydrolase

3.9 Spectral changes for reactions of protocatechuate branch of β-ketoadipate pathway

The absorption spectra of substrates and products of the protocatechuate branch of β-ketoadipate pathway were recorded in the presence of successive additions of the purified enzymes identified above (see *Materials and Methods*). During the experiment the same baseline of buffer only was always used. The visible spectrum of protocatechuic acid showed two peaks at approximately 250 and 290 nm. By adding the purified protocatechuate 3,4-dioxygenase (NRRL_01405), these peaks disappeared and a species with a broad absorbance maximum at 260 nm was generated (Figure 3.12). This corresponds to the conversion of protocatechuic acid to β-carboxy-cis,cis-muconate[18, 60]. The peak at 260 nm disappeared after adding β-carboxymuconate cyclase (NRRL3_02586) to the assay solution, which is consistent with production of β-carboxymuconolactone as observed by Cain *et al.*[18] (Figure 3.12). The absorbance decreased and was shifted to the far UV by addition of β-carboxymuconolactone hydrolase, consistent with the spectral changes reported by Cain *et al.*for the conversion of β-carboxymuconolactone to β-ketoadipate (Figure 3.12).

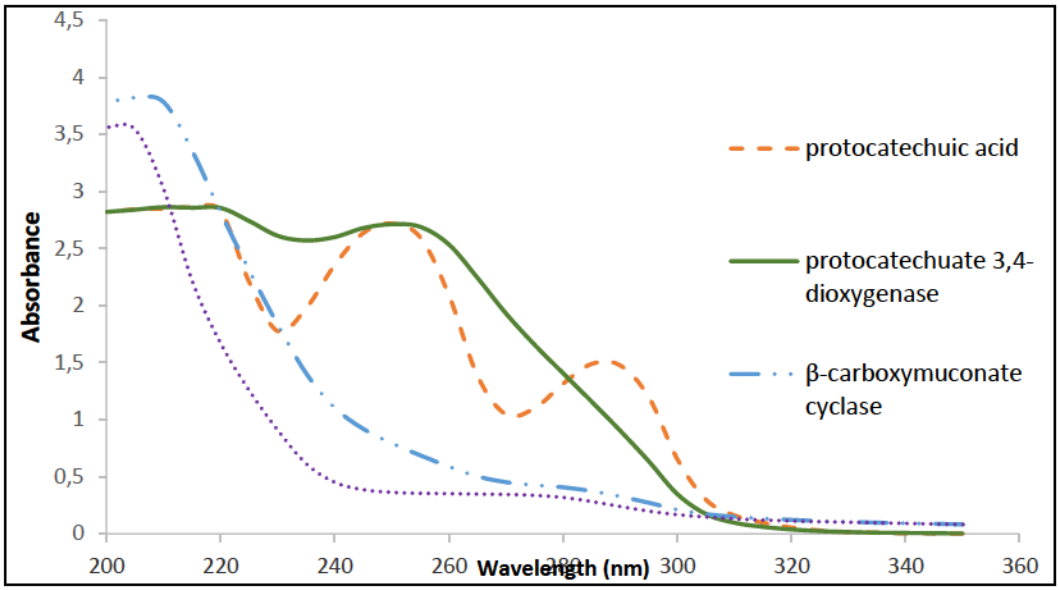


Figure 3.12: Ultraviolet absorption spectra observed in enzymatic reaction mixture: for protocatechuic acid in orange, after adding protocatechuate 3,4-dioxygenase in dark green, after adding β-carboxymuconate cyclase in blue and after adding β-carboxymuconolactone hydrolase in purple. After the addition of each enzyme, spectra were recorded (not shown) until they ceased changing and then the spectrum shown was recorded before the addition of the next enzyme. The spectra were recorded using a buffer only baseline.

3.10 Estimating native molecular masses of purified enzymes

3.10.1 Size exclusion chromatography (SEC)

The estimation of the native molecular weights of the purified proteins encoded by NRRL3_10507, NRRL3_04788 and NRRL3_01409, and by extension their oligomeric states, was carried out using data from chromatography on a calibrated Superdex 75 10/300 GL gel filtration column (Figure 3.13), as described in section 2.11.2. The estimated molecular weights of 37 and 50 kDa respectively for NRRL_10507 and NRRL3_04788 suggest the dimeric form of these protein based on their predicted monomeric molecular weights of 17 kDa and 27.62 kDa, respectively (Table 3.7). The estimated molecular weight of 67 kDa for NRRL3_01409 suggests a monomeric form of this protein based on the predicted molecular weight of 66 kDa (Table 3.7).

***A. niger* Protein**	**Theoretical Molecular Weight (kDa)**	**Estimated Molecular Weight (kDa)**	**Oligomeric State**
Muconoloactone isomerase	17	37	Dimeric
β-ketoadipate enol-lactone hydrolase	27.62	50	Dimeric
β-carboxymuconolactone hydrolase	64	66	Monomeric

Table 3.7: Molecular weights and oligomeric state of purified proteins

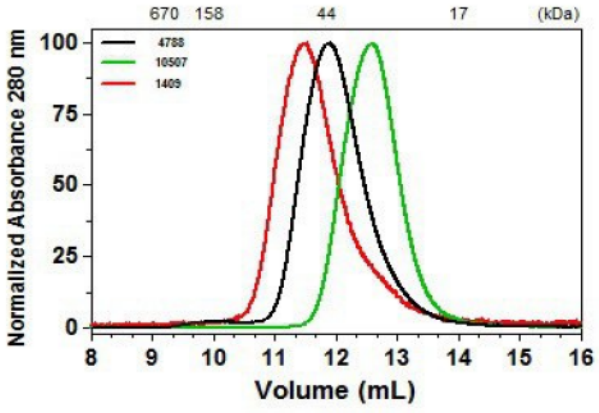


Figure 3.13: Elution volumes observed during estimation of molecular weight of NRRL3-10507, NRRL3-4788 and NRRL3-1409 and oligomeric state by size exclusion chromatography (SEC)

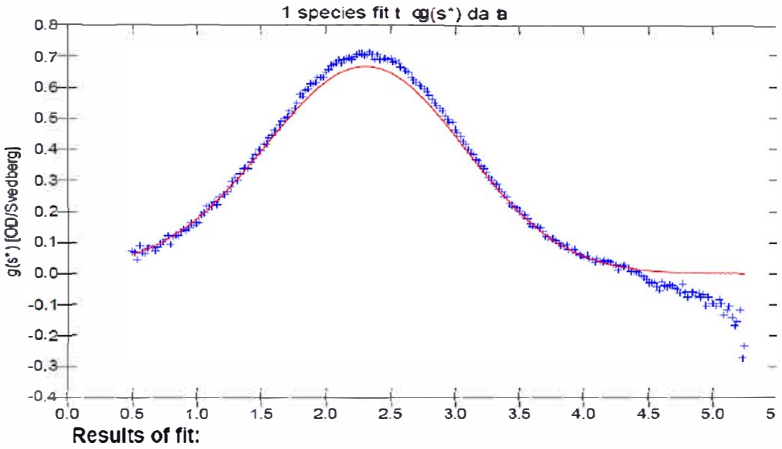
3.10.2 Analytical Ultracentrifugation (AUC)

Analytical ultracentrifugation was carried out as described in section 2.11.1. Figure 3.14 shows the DCDT analysis results for the sedimentation velocity experiment of NRRL3_10507, NRRL3_04788 and NRRL3_01409. For NRRL3_10507 a single sedimenting species with estimated molecular mass of 34 kDa is observed, which is about twice of the estimated monomeric molecular mass of 17 kDa (Table 3.7). NRRL3_04788 sedimented as a single species with estimated molecular mass of 49 kDa, suggesting the dimeric form of this enzyme. As for NRRL3_01409, it sedimented as a single species with estimated molecular mass of 65 kDa which corresponds to the monomeric form of this enzyme (Table 3.8). These results are consistent with the results from size exclusion chromatography (Table 3.7).

A. niger Protein	Theoretical Molecular Weight (kDa)	Estimated Molecular Weight (kDa)
NRRL3_10507	17	2 * 17000
NRRL3_04788	27.62	2 * 27000
NRRL3_01409	64	1 * 65000

Table 3.8: Analytical ultracentrifuge result for muconoloactone isomerase, β-ketoadipate enol-lactone hydrolase and β-carboxymuconolactone hydrolase

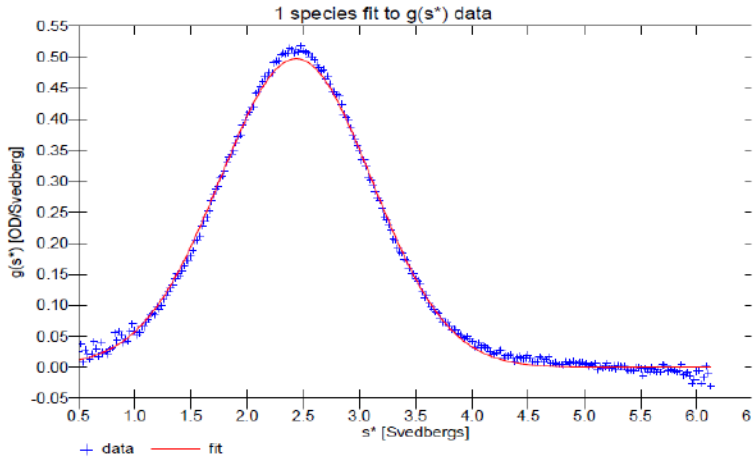
A



Results of fit:

Parameter	Best Value	99% Confidence Limits	Starting Value
Co(1) (OD)	1.2678	1.2451, 1.2910	1.3038
s20,w(1) (S)	2.401	2.385, 2.417	2.340
M(1) (kDa)	32.93	31.38, 34.46	34.05

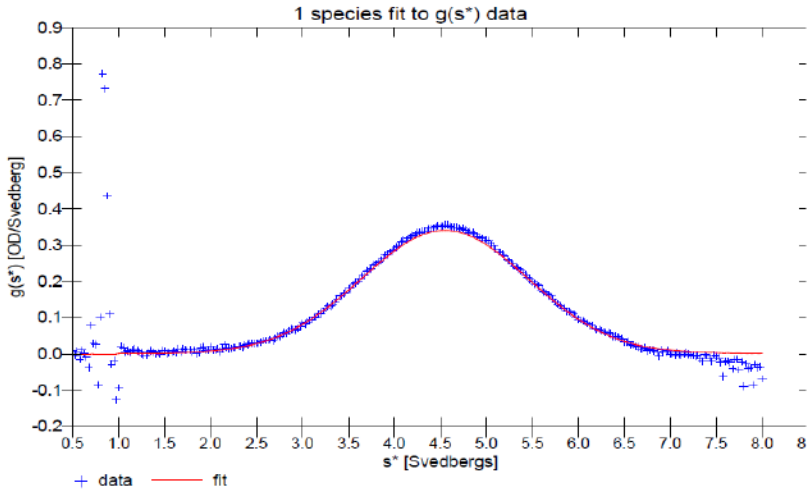
B



Results of fit:

Parameter	Best Value	99% Confidence Limits	Starting Value
Co(1) (OD)	0.8229	0.8126, 0.8338	0.7919
s(1) (S)	2.502	2.492, 2.512	2.480
M(1) (kDa)	46.53	45.02, 47.97	55.74

C



Results of fit:

Parameter	Best Value	99% Confidence Limits	Starting Value
Co(1) (OD)	0.7810	0.7564, 0.8050	0.7944
s(1) (S)	4.590	4.562, 4.619	4.550
M(1) (kDa)	65.05	59.84, 70.55	63.99

Figure 3.14: Sedimentation velocity analytical ultracentrifugation: of purified A NRRL3_10507 muconoloactone isomerase, B NRRL3_04788 β-ketoadipate enol-lactone hydrolase and C NRRL3_01409 β-carboxymuconolactone hydrolase. The DCDT analysis of the AUC data are shown, with the upper panel showing the g(s*) plot and 99% confidence interval.

4 Discussion

The goals of this research were to express and assign functions to genes proposed to be involved in the β-ketoadipate pathways for catechol and protocatechuate in *Aspergillus niger*. Beta-ketoadipate is one of the central intracellular metabolites in fungi and bacteria that metabolize catechol and protocatechuate and convert them to metabolites of the tricarboxylic acid cycle. Despite extensive studies in bacteria, fungal species are less well studied. In particular, although many fungal genes have been annotated in genome projects as catalyzing these activities, few studies have actually isolated the corresponding proteins and shown them to have the predicted functional properties. The results are expected to improve significantly our knowledge of fungal pathways and enzymes involved in biodegradation of aromatic compounds, and support future studies, such as on gene regulation, which may benefit from this information.

A. niger was chosen for the following reasons:

a) Its genome has been fully sequenced and is publicly available.
b) A variety of its enzymes and activities are used in industry and it can readily be genetically manipulated.
c) Expertise with *A. niger* is available in the Centre for Structural Genomics at Concordia University.
d) Metabolites and enzyme activities of the β-ketoadipate pathway in *A. niger* have been reported by Cain *et al.*[18, 23, 24] although at a time when sequence information was not available to correlate with particular genes.

The approach that was taken was to identify genes in the *A.niger* genome, either from the genomic sequence annotation or BLAST comparisons with genes encoding known enzyme activities, amplify them from cDNA using PCR, clone them into a bacterial expression vector and then after expression purify and characterize the resulting proteins with respect to activity and some other properties.

The target sequences in the *A. niger* genome were selected by querying the *A. niger* genome with sequences of proteins from *A. nidulans,* proposed to be involved in the β-ketoadipate pathway via genomics and proteomics experiments[21] , as well as sequences of known enzymes from *P. putida and A. calcoaceticus* β-ketoadipate pathways. In total, eleven sequences were chosen (Table 3.2) corresponding to the enzymes involved in down-stream reactions of the β-ketoadipate pathway. Some of these sequences have low identity to those of the published enzymes especially those from prokaryotes, and in some cases

there is more than one candidate. PCR amplification was carried out using using *A. niger* cDNA and subsequently cloned into the pLATE11 vector using the ligation independent aLICator LIC Cloning kit. All of the chosen genes were successfully amplified and cloned. Each plasmid was transformed into *E. coli BL21(DE3*) and then tested for expression using screening by SDS-PAGE. Six genes were successfully expressed and from them one was insoluble and five were the soluble proteins (Table 3.3). These proteins were then tested for β-ketoadipate pathway enzyme activities first in crude extract and after purification.

4.1 Catechol branch of the β-ketoadipate pathway

The gene, *catB*, from *Pseudomonas putida*[57] used as a query sequence in a BLAST search against the *A. niger* genome and NRRL3_11120 was identified as a gene possibly encoding MLEI activity (Table 3.2). This gene was successfully expressed as a soluble protein (Table 3.3) however its activity should be further investigated in future studies.

Based on bioinformatics comparisons, NRRL3_10507 and NRRL3_04788 were predicted to possibly have muconolactone isomerase and β-ketoadipate enol lactone hydrolase activities, respectively (Figure 3.10). Using enzyme activity assays similar to those originally reported for the orthologous proteins from *P. putida*[14] resulted in confirmation of muconolactone isomerase activity and β-ketoadipate enol lactone hydrolase activity for NRRL3_10507 and NRRL3_04788 proteins respectively. The activity of muconolactone isomerase was confirmed by monitoring the disappearance of muconolactone at 230 nm and 25 °C in the presence of excess β-ketoadipate enol-lactone hydrolase. In the assay solution the product of muconolactone isomerase exist in two tautomeric forms and one of these forms absorbs considerably at 230 nm due to its furan ring. On the other hand β-ketoadipate enol-lactone hydrolase converts this product to β-ketoadipate which is transparent at 230 nm[20]. Therefore,the decrease in absorbance observed in this assay set-up (Figure 3.3), but only in the presence of the hydrolase, is consistent with the isomerase activity. The decrease in absorbance at 230 nm only upon the addition extract of cells expressing NRRL3_04788 suggests that it is the β-ketoadipate enol-lactone hydrolase.

The bioinformatics comparisons also suggested the possibility of β-ketoadipate enol lactone hydrolase activity for NRRL3_01409. However, its predicted molecular weight is quite different from NRRL3_04788, which as explained above, has activity. Using NRRL3_1409-encoded enzyme in the assay activities described above did not result in any changes in the absorbance. Therefore, this enzyme

does not have the predicted activity in our experiments. As described below, this enzyme has appears to be the protocatechuate branch β-carboxymuconolactone hydrolase enzyme observed by R. Thatcher *et al*[23]. After confirming the activity in crude extracts, three-step protein purifications were carried successfully for the NRRL3_10507 and NRRL3_04788 encoded enzymes.

The muconolactone isomerase encoded by NRRL3_10507, was purified with a 7.1fold increase in purity (Figure 3.7). Also, it was determined to be a dimer with a mass of 37000 Da by combining SDS polyacrylamide gel electrophoresis, which showed a monomer mass of 170000 Da, and Superdex S-300 chromatography which showed a single species migrating at 37000 Da. The analytical ultra-centrifuge (AUC) results indicated a very similar overall mass of 34000 Da. L. N. Ornston *et al.* [20]purified and characterized this enzyme from the catechol branch of the β-ketoadipate pathway of *P. putida*. Based on their studies this enzyme is a decamer with a molecular weight of 93000 Da and a subunit mass of 11000 Da[61]. The molecular weight and subunit composition of the purified NRRL3_10507 is considerably different than the decamer muconolactone isomerase from *Pseudomonas putida*, and a BLAST comparison of the amino acid sequences show no significant similarity. However, the orthologue from *A. nidulans,* AN4061, predicted to have multimeric NTF2 like protein domains based on InterPro-Scan classifications which suggests a new class of multimeric muconolactone isomerases. Moreover, some homologous proteins found in Dikarya fungi (133 species) and Ascomycota (121 species)[21].

Interestingly we were not able to find any homologs of this protein in the SwissProt database, therefore more investigation is needed to determine domains and catalytic sites of this protein.

The putative β-ketoadipate enol lactone hydrolase, encoded by NRRL3_04877, was purified 9.7fold and a significant increase in its specific activity (Fig 3.8). The purified protein appears to be a dimer with a mass of 50000 Da by Superdex S-300 chromatography and a subunit molecular weight of 27.6 kDa by SDS polyacrylamide gel electrophoresis. The AUC result showing a single species with a molecular mass of 49 kDa. This enzyme is larger than the dimeric β-ketoadipate enol lactone hydrolase from *P. putida* with a molecular weight of 33000 Da as reported by L. N. Ornston[20]. The NRRL3 annotation of of β-ketoadipate enol-lactone hydrolase indicates that the enzyme has the Alpha/Beta hydrolase fold. A BLAST comparison detected no significant similarity with the *P. putida* enzyme sequence, but a BLAST search of the SwissProt database indicates 28% identity to 3-oxoadipate enol-lactonase 1 from *Actinobacter baylyi*[62].

This result is consistent with the study that has been reported by Martin on the orthologue from *A. nidulans*. He has reported that AN4531 codes a protein with 510 amino acid residues with Alpha/Beta hydrolase fold and theoretical molecular weight 56 kDa and experimental molecular weight 27 kDa[21].

4.2 Protocatechuate branch of the β-ketoadipate pathway

The protocatechuate branch of the β-ketoadipate pathway involves a lactonization step where the ring cleavage product, β-carboxymuconate, is cyclized to β-carboxymuconolactone, which is then hydrolyzed and decarboxylated to β-ketoadipate, reactions that are specific to fungi[18]. The gene product of NRRL3_02586 was predicted to have the β-carboxymuconate cyclase activity of the protocatechuate branch on the basis of a sequence comparison with the *A. nidulans* orthologue, AN1151, which was proposed on the basis of proteomic studies. In this study the gene replacement mutant Δ1151 did not grow in benzoate medium and showed the acumulatuoin of the 3-carboxy-ciscis-muconate which suggests the essential role of this gene in benzoate usage as a carbon source[21].

This enzyme has been purified and to some extent characterized by Cain *et al.* but they did not report the sequence of the corresponding gene[18, 23, 24]. The expressed protein encoded by NRRL3_02586 was partially purified and its activity confirmed by monitoring the disappearance of β-carboxy-*cis,cis*-muconate, which has a strong absorption at 260 nm (Fig 3.6B). The isolated enzyme preparation also had a pH optimum similar to that reported by Cain's group[23] (Fig 3.10).

Initially it was predicted that β-carboxymuconolactone hydrolase activity might be encoded by NRRL3_08340, which is the *A. niger* orthologue of a gene putatively identified in *A. nidulans* on the basis of proteomic studies (AN5232). In this study the gene replacement mutant ΔAN5232 did not grow in benzoate medium and showed the acumulatuoin of the 3-carboxymuconalactone which suggests the essential role of this gene in benzoate usage as a carbon source[21]. β-carboxymuconolactone hydrolase has been purified previously from *Aspergillus* niger and assayed by R.Thatcher *et al*[24]. They reported that, the product of the 3,4-dihydroxybenzoate cleavage reaction, β-carboxymuconolactone, absorbs at 230: by adding β-carboxymuconolactone hydrolase to the assay solution the decrease in absorbance confirms β-carboxymuconolactone hydrolase activity. However, an extract of cells expressing NRRL3_08340 resulted in no change in absorbance. Therefore, this protein does not have the predicted activity in our experiments.

As reported by R.Thatcher *et al.*the enzyme they isolated had both decarboxylation and hydrolysis activities and an estimated molecular weight on polyacryamide gel electrophoresis of 54 kDa[18, 24]. However, the NRRL3_08340 encoded protein has a predicted molecular weight of about 16 kDa which is only about a third of the size of their previously characterized enzyme. As mentioned in the previous section on the catechol branch activities, the amino acid sequence encoded by NRRL3_01409 showed some sequence similarity to bacterial β-ketoadipate enol lactone hydrolase but did not have that predicted activity in our experiments. The predicted and observed molecular weight of the encoded protein is 60 kDa, which is close to the molecular weight of the enzyme studied by R. Thatcher *et al.* Using NRRL3_01409 in an assay solution containing β-carboxymuconolactone as the substrate, showed decrease in absorbance at 230 nm and confirmed the β-carboxymuconolactone hydrolase activity of NRRL3_01409 protein (Fig 3.6C). This result is consistent with Cain's spectral changes assay (Fig 7)[18] as well as our spectral changes result (Fig.12).

The expressed protein from NRRL3_1409 went through a 4 steps purification procedure. Despite the minimal increase in purity by SDS-PAGE, it showed a decrease in specific activity during purification (Fig 3.11. The decrease in specific activity may indicate some instability (as reported by Thatcher and Cain) that should be further examined. Previously Thatcher and Cain showed that the β-carboxymuconolactone hydrolase is a monomer with the molecular weight of 54000 Da[24]. The protein isolated in this study consisted of a monomer with a mass of 66000 Da by SDS-polyacrylamide gel electrophoresis and a very similar estimated mass by Superdex S-300 chromatography. AUC experiments showed a result similar to SEC. The NRRL3 annotation of β-carboxymuconolactone hydrolase indicated that the enzyme has the Alpha/Beta hydrolase fold and dimeric alpha-beta barrel. A part of the protein has an unknown domain that should be further investigated.

In summary, this study assigned 5 previously uncharacterised genes to downstream reactions of the β-ketoadipate pathway in *A. niger*, after expression in *E. coli*, enzymatic assay, and purification. The sequence (NRRL3_01409) of β-carboxymuconolactone hydrolase-decarboxylase is the first reported for this enzyme. This project provided novel information about some molecular properties of β-carboxymuconate hydrolase (NRRL3_01409), along with new information of some properties of two enzymes muconolactone isomerase (NRRL3_10507) and β-ketoadipate enol lactone hydrolase (NRRL3_04788).

The findings reported here increase our knowledge about fungal enzymes involved in degradation of aromatic compounds and could be useful for the development of engineered strains for efficient degradation of aromatic compounds in bio-refinery and bioremediation applications.

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